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A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Ecological impacts of *Bt* transgenic cabbage expressing Cry1Ac1 protein on non-target arthropod species

Cry1Ac1 단백질을 발현하는 양배추의 비표적 절지동물 종들에 미치는 생태적 영향

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February 2017

Ecological impacts of *Bt* transgenic cabbage expressing Cry1Ac1 protein on non-target arthropod species

UNDER THE DIRECTION OF ADVISER JOON-HO LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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Abstract

With the rapid advancement of molecular technique, transgenic crops that have much kind of traits, such as pest resistance and herbicide resistance were developed. These beneficial traits have helped reduce the damage from pests or cost of herbicide use. As a result, transgenic crops have been commercially cultivated since 1996, and those have been increasing dramatically over the last decades. However, concerns remain about the potential adverse effects of such crops on ecosystems. Especially, among the organisms that interact closely with transgenic crops, primary consumers (herbivores) and upper trophic level arthropods (predators and parasitoids) can be affected by crops. Therefore, the "Cartagena Protocol on Biosafety" and the "Living Modified Organisms Act" stipulate that the scientific and reasonable environmental risk assessment should be conducted. In the present study deals mainly with the environmental risk assessment of transgenic *Bt* cabbage expressing the insecticidal Cry1Ac1 protein which was aimed at controlling lepidopteran pests. This study comprised three parts. (1) Levels of *Bt* protein in herbivorous and predatory arthropods in fields of *Bt* cabbage. (2) Effects of *Bt* cabbage on the survival and growth of the wolf spider, *Pardosa astrigera*. (3) Effects of transgenic cabbage expressing Cry1Ac1 protein on target pests and the non-target arthropod community under field

conditions. Biosafety of transgenic crops are open to interpretation by scientific technique, food problem and social identity. This study may help to understand these problems.

Key words:

Arthropod, community, environmental risk assessment, life history trait, transgenic crop

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Chapter I.

General Introduction

1.1. Advent of transgenic crops

For the past centuries the breeding by crossing hybridization was only technique for the introducing a genetic trait in crops. With this technique, scientists tried to create new genetic traits that show better performances. Although this method has been achieved good results in development of crop quality and productivity, there are some bad points to perform this. For example, it requires a lot of time that is depending on the generation time of the species of each plant. In addition, there is also a disadvantage on what scientists can introduce new genetic trait because each plant has limited gene pool.

The 20th century was a time that new techniques including mutations by chemicals, treatment of bioactive rays and the culture of anther and ovule become available (National Research Council, 1989). Most of all, the transformation is the most innovative molecular biology technique in this century. Genetic modification (GM) is the introduction of beneficial traits to and organisms by making changes to its DNA sequences through intervention at the molecular level. This technique is performed through the insertion of a segment of DNA containing the biological information that including active trait into the genome of a crop plant and this process can be possible with bacteria or viruses as vectors, or by the forced insertion (gene-gun inoculation) of the DNA segment directly into plant cells.

According to James (2014), transgenic crops including maze and soybean have been commercially available for agricultural management since 1996, and the area worldwide in which transgenic crops are grown exceeded 18.5 million ha in 2014. Farmers have adopted the use of these transgenic crops because this practice reduces the need for agricultural chemicals while also increasing crop quality and yields (Betz et al., 2000; Clark et al., 2005).

In total, 12 of transgenic crop plants (corn, soybean, cotton, rape, sugar cane, alfalfa, pumpkin, tomato, paprika, poplar and eggplant) are commercially available globally. Among them, 82% of soybean, 68% of cotton, 30% of corn and 25% of canola are transgenic crops. The traits of transgenic crops are mainly on herbicide resistance, pest resistance, plant virus disease resistance and amylose free, and especially, the number of transgenic crops that have both traits; herbicide resistance and pest resistance are increasing.

On the domestic side, there are various pest resistance studies using insecticidal *Bt* gene have been conducted (Park et al. 2000; Roh et al. 2007), and also new pest resistance crops that developed by transgenic technique is in progress. For example, Park et al. (2000) had developed the diamondback moth (*Plutella xylostella*) resistant Chinese cabbage, and Nam et al. (2009) had also developed the rice leaf roller (*Cnaphalocrocis medinalis*) resistant rice. Among the cruciferous crops, the transgenic research on Chinese cabbage is the most

active. Although there are many kind of transgenic Chinese cabbage including herbicide resistance, pest resistance and containing tocopherol is now investigating, research scale of cabbage is relatively small when compared to Chinese cabbage.

1.2. Environmental risk assessment of insect resistance transgenic crops using arthropod species

The potential influence of insect-resistant transgenic crops on non-target organisms, including primary consumers and predators, has been an important issue when assessing possible environmental risks from those plants. For example, *Bt* toxins can be transferred not only directly to crop-fed herbivores but also indirectly to their predators via trophic pathways (Carpenter 2011). Therefore, transgenic crop varieties should be conducted rigorous environmental risk assessment before commercial use. Because of this, risk assessments have been conducted to address concerns about environmental safety and any negative effects of *Bt* crops on the non-target food chain (Naranjo 2009; Devos et al. 2012).

The environmental risk assessment of transgenic crops using arthropod species is planned to answer the question about the ecological effect of introducing such transgenic crops on arthropod species, and includes three main stages; 1) problem formulation, 2) risk hypothesis, and 3) appropriate tier-test for their evaluation (Romeis et al. 2008). The problem formulation is very important and crucial early step to identify the areas of prime concern or unidentified ecological risks. This defines the scope of the risk assessment by hypothesis that is subsequently addressed in an appropriate tiered scheme of ecological risk

assessment (Garcia-Alonso et al. 2006; Rose 2007; Hilbeck et al. 2011). In the tiered scheme of ecological risk assessment, early-tier laboratory tests are designed to investigate specific endpoint (e.g. life history traits; body mass, development, fecundity etc.) under laboratory conditions using protein contents that are generally several times higher than those concentration in field. These worst-case exposure conditions show meaningful results because the insecticidal proteins can be directly exposure. Although early-tier tests are not reflecting actual exposure condition realistically, it improves a possibility of detection of hazard, and also to increase confidence in ecotoxicological safety claims. Next, higher-tier tests are conducted under semi-field (greenhouse) or open field conditions. These trials more realistically represented what arthropods is exposure to under field condition. The results of such trials can, however, be difficult to interpret due to unexpected environmental variables. So far, earlier-tier tests are more valuable and reliable to assess the environmental risks because it is surely conducted under highly controlled experimental setting, and may provide meaningful data and experimental methods (Romeis et al. 2011).

1.3. Insecticidal transgenic cabbage expressing Cry1Ac1 protein

A transgenic line (C95) of cabbage (*Brassica oleracea* var. *capitata*) was developed from AD126, a non-transgenic control line, to contain *cry1Ac1* gene obtained from a soil bacterium, *Bacillus thuringiensis* (GenBank Accession No. AY126450; Park et al. 2003). Expression is under the control of the cauliflower mosaic virus 35S promoter and the *nos* terminator. The transgenic line also has *neomycin phosphotransferase II* to impart kanamycin resistance as a selection marker (Harn et al. 2011). An Incubating system for breeding diamondback moth (*Plutella xylostella*) established so that the resistant transgenic cabbages were selected at the seedling stage of cabbage in the chamber. T_1 seeds of the event line were resistant to the *P. xylostella* at a rate of 100% resistance (Fig 1-1, Kim 2014). The selected cabbages were self-crossed and the T_2 cabbages resistant to *P. xylostella* were also selected. Plants of these transgenic and non-transgenic cabbage lines were provided by the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea.

Fig 1-1. Resistance of transgenic *Bt* cabbage to the diamond back moth, *Plutella xylostella*. Transgenic cabbages were not damaged by *P. xylostella* while the control cabbages were totally damaged by *P. xylostella.*

1.4. Objective of this study

Regarding the environmental safety of transgenic crops, large and diverse numbers of people; farmers, consumers, scientists, agricultural chemical companies and government have been a heated discussion, and they have opposite opinions. In this confrontational situation, clear independent fact and opinions based on the scientific and rationalistic inquiries are needed.

In the case of South Korea, there is no transgenic crop that passed a risk assessment procedure for commercial use. Namely, preparations for the upcoming the commercialization time of transgenic crops, e.g., technical skills and risk assessment system are deficient when compared to other advanced countries. In this regard, it is very important to standardize safety criteria via well-organized environmental risk assessment scheme. Because, the environmental risk assessment is almost a necessity for the decision to cultivate these crops commercially.

Therefore, this study largely comprises three parts based on the tiered scheme of ecological risk assessment. First, field monitoring level: For establishing the risk hypothesis, we examined the levels of Cry1Ac1 protein in *Bt* cabbage and in the arthropod species that are exposed to those plants under field conditions. Moreover, we used the exposure levels that we found for *Bt* protein in arthropod species as a tool for screening competent surrogate species in order to

conduct non-target risk assessments that reflect realistic conditions in a cabbage field.

Second, tritrophic level: As an extended laboratory study, we conducted a tritrophic bioassay to evaluate the ecotoxicological impacts that *Bt* cabbage (*Brassica oleracea* var. *capitata*) expressing Cry1Ac1 protein might have on the wolf spider (*Pardosa astrigera*), a non-target generalist predator.

Third, community level: As an open field study, we investigated how transgenic *Bt* cabbage expressing the insecticidal Cry1Ac1 protein affects two target Lepidoptera species, *Plutella xylostella* (Plutellidae) and *Pieris rapae* (Pieridae), as well as the structure of the local non-target arthropod community.

Our studies provide a comprehensive risk study system and independent data relevant to the environmental risk assessment of *Bt* transgenic cabbage and present basic information about the ecological effects of this crop on arthropod species.

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Chapter II.

Literature review

2.1. Effects of transgenic crops expressing Cry1Ac protein on target arthropods

The main purpose of transgenic *Bt* crops is to control the target pests. The effectiveness of those crops needs to be examined by detailed scientific tests before conducting the environmental risk studies (Romeis et al. 2008). Many research articles have addressed the efficacy of number of transgenic crops (broccoli, cabbage, cotton, potato and rice) (Table 2-1). Those include laboratory and field studies that investigated specific endpoints such as mortality, development and population. Generally, many studies showed that the pest control efficacy of those *Bt* plants was very high under both laboratory and field conditions.

Table 2-1. Effects of transgenic crops expressing Cry1Ac protein on target arthropod species under laboratory and field conditions.

Crops	Target species	Experimental condition	Measurement	Effects	References
Broccoli	*Plutella xylostella*	Laboratory	Cry1Ac contents of *P. xylostella*, mortality	Rapid mortality of *P.xylostella* larvae	Cao et al. 2002
Cabbage	*Plutella xylostella*	Laboratory	mortality	Complete mortality of larvae from susceptible *P.xylostella*	Cao et al. 2005
Cabbage	*Plutella xylostella, Pieris rapae*	Field	Abundance	Lower abundance of *P. xylostella* and *P. rapae*	Kim et al. 2015
Cotton	*Spodoptera exigua*	Laboratory	Development, food utilization and population performance	Longer larval period, lower pupal weight and lower food consumption	Wu et al. 2009
Oilseed rape	*Plutella xylostella*	Greenhouse	Cry1Ac contents of plant	Cry1Ac concentration increased significantly as the leaf age	Wei et al. 2005
Poplar	*Apocheima cinerarius*	Field	Abundance, percentage of the leaves damaged.	Less abundance of *A. cinerarius* and lower percentage of leaves damaged than control poplar.	Hu et al. 2000

Table 2-1. Continued.

Crops	Target species	Experimental condition	Measurement	Effects	References
Potato	*Phthorimaea operculella*	Field	Development	Inhibition of growth rates were inhibited	Davidson et al. 2002
Potato	*Phthorimaea operculella*	Field	Number of mine, percentage of damaged tuber, abundance	Tuber of *Bt* potato had lower mine damage than control potato. Abundance of *P. operculella* in *Bt* potato was much smaller than control potato	Davidson et al. 2006
Rice	*Scirpophaga incertulas*	Laboratory	Mortality	Showed higher *S. incertulas* mortality which was fed with *Bt* rice	Khanna and Raina 2002
Rice	*Cnaphalocrocis medinalis, Naranga aenescens, Parnara guttata, Mythimna separate*	Laboratory and field	Mortality, percentage of affected plants and leaves from pests in field	*Bt* rice showed no symptoms of damage, whereas nontransgenic control plants were severely damaged by *C. medinalis*.	Kim et al. 2009

2.2. Effects of transgenic crops expressing Cry1Ac protein on non-target arthropods under laboratory conditions

Generally, the risk assessment study follows a tiered scheme that starts with worst-case and direct-exposure laboratory studies. Those studies have a high reliability to show adverse effects on non-target test species (Romeis et al. 2011). A number of meaningful data that can be carried out for the risk assessment may already exist in the previously conducted earlier-tier studies (Table 2-2). Table 2-2 summarizes the outcomes of the effects of Cry1Ac crops on non-target arthropods including parasitic wasps, predatory lady beetle, wolf spider and lacewing, and herbivory aphids, Noctuidae species and thrips. However, in the case of lepidopteran larvae, *Bombyx mori* showed decreased survival and growth. As a result, transgenic crops expressing Cry1Ac protein show that there is no negative effect on non-target arthropods except lepidopteran herbivore species in many cases.

For the clear guidance, such data from laboratory-risk studies assists the crop developers and researchers. WHO (2008) suggested the four qualitative criteria for chemical exposure assessment as follows; 1) Appropriateness: relevance and applicability of data for specific exposure assessment, 2) Accuracy: the compatibility of calculated or modeled values with the true values that are

intended to represent, 3) Integrity: integrity of the data investigated and reported, and 4) Transparency: the degree of accuracy and completeness of all data, methodology and hypothesis.

Table 2-2. Effects of *Bt* (Cry1Ac) crops on non-target arthropod species under laboratory conditions.

Crops	Target species	Measurement	Effects	Reference
Chinese cabbage	*Mamestra brassicae*, *Microplitis mediator*	Parasitization rate, development, Cocoon weight, adult emergence rate	No effect	Kim et al. 2008
Chinese cabbage	*Bombyx mori*	Survival, body weight	Decreased survival rate and weight of *B.mori* which fed with *Bt* Chinese cabbage pollen contained artificial diet	Kim et al. 2008
Corn	*Rhopalosiphum maidis*, *Cotesia margiventris*	Abundance, survival rate	Higher abundances of aphids on *Bt* plants resulted in an increased production of honeydew. Parasitoid lived longer and parasitized more pests in the presence of aphid-infested *Bt* maize.	Faria et al. 2007
Cotton	*Aphis gossypii*	Honeydew analysis, nymphal developmental time and life history	No effect. None of the aphid samples contained *Bt* protein.	Lawo et al. 2009
Cotton	*Aphis mellifera*	Mortality, SOD(superoxide dismutase)activity	No effect	Liu et al. 2009
Cotton	*Aphis gossypii*, *Chrysopa pallens*	Mortality, development of the larval stages, pupal body mass, adult sex ratio, fecundity, and egg viability	*Bt* cotton originated aphid prey has no adverse impact on survival, development, and fecundity of *C. pallens*	Guo et al. 2008

Table 2-2. Continued.

Crops	Target species	Measurement	Effects	Reference
Cabbage	*Pardosa astrigera*	Survival, development, adult weight	No effect	Kim et al. 2016
Cotton	*Coleomegilla maculata, Trichoplusia ni*	Survival, development, adult weight, fecundity	No effect	Li et al. 2011
Cotton	*Apis mellifera*	Mortality, feeding behavior	No lethal risk for honey bees. Bees consumed significantly less *Bt* cotton pollen than in the control cotton.	Han et al. 2010
Cotton	*Pseudoplusia includens, Cotesia marginiventris, Copidosoma floridanum*	Development, longevity, pupal weight	Feeding on *Bt* cotton by *P.includens* affected the survival and development of the two hymenopteran endoparasitoids.	Baur and Boethel 2003
Cotton	*Euseius concordis, Neoseiulus californicus*	Reproduction	No effect	Castro et al. 2013
Cotton	*Spodoptera exigua, P. maculiventris*	*Bt* contents of insects, survivorship, development	No effect	Toress and Ruberson 2008
Oilseed rape	*Athalia rosae*	Mortality, larval development, weight	No effect	Howald et al. 2003
Oilseed rape	*Plutella xylostella, Cotesia plutellae*	Parasitoids development, percentage of parasitism	No effect	Schuler et al. 2004

Table 2-2. Continued.

Crops	Target species	Measurement	Effects	Reference
Rice	*Stenchaetothrips biformis*	Larval and pupal period, longevity, reproduction, body contents of Cry1Ac	Longer larval, pupal development and preoviposition durations of *S. biformis* in *Bt* rice.	Akhtar et al. 2010
Purified protein	*Cheilomenes sexmaculatus*	Larval and pupal development, survival, weight and adult emergence	Decreased larval survival and adult emergence from direct exposure through Cry1Ac protein contained artificial diet. No effect of indirect exposure through aphid feeding.	Dhillon et al. 2009
Purified protein	*Chrysoperla carnea*	Larval and pupal development, survival and adult weight	No effect	Lawo and Romeis 2008

2.3. Effects of transgenic crops expressing Cry1Ac protein on non-target arthropods under field conditions

Crop plants have a major influence on communities of overall arthropods in field which are fundamental to many functions of ecological systems, such as habitat provision and mobilization of nutrients. These functions are definitely affecting both the abundances of arthropods and their diversity. A general risk hypothesis concerning non-target arthropods effects of pest-resistant transgenic crop is; "The expressed *Bt* toxin is not harm on non-target arthropods at the true level present in the field" (Raybould 2007). Although field-risk studies are longer in duration than laboratory study and cannot easily be interpreted, those would be very reliable if high-quality and long-term data are accumulated because these data sources clearly demonstrate ecological food web interactions.

In the table 5-3 summarizes the outcomes of the effects of Cry1Ac crops on non-target arthropods species under field conditions. Most of these studies focused on abundance and local community structure of non-target arthropods in crop fields (Table 2-3). Generally, Cry1Ac-transgenic crops did not adversely affect non-target arthropod species or community. Rather, higher abundances of natural enemies were found in *Bt* cotton (Hgerty et al. 2005; Head et al. 2005).

Meanwhile, Akhtar et al. (2010) showed that the abundance of thrip species were significantly lower in the *Bt* rice plots than in the non-transgenic control rice plots.

Table 2-3. Effects of *Bt* (Cry1Ac) crops on non-target arthropod species under field conditions.

Crops	Target species	Experimental condition	Measurement	Reference
Cabbage	Pests and non-target arthropods	Abundance, community structure	No effect on non-target arthropod community	Kim et al. 2015
Corn	Coleopteran species	Abundance	No differences in total abundance of coleopteran species. Negative effects of *Bt* corn were showed in 3 of 39 species.	Floate et al. 2007
Corn	Hemipteran species	Abundance	No effect	Rauschen et al. 2008
Cotton	Sucking insects, foliage feeders and predators	Abundance, time of first appearance	Similar abundance and no difference in time of first appearance of non-target species	Mann et al. 2010
Cotton	*Chrysoperla carnea, Orius tristicolor*	Abundance	No natural enemy abundance differences between *Bt* and non-*Bt* cotton	Sisterson et al. 2007
Cotton	*clubiona sp., Neoscona sp., Cheilomenes sexmaculatus, Chrysoperla carnea, Campoletis chlorideae*	Abundance	No effect on natural enemy (spiders, green lacewing, lady bird) abundance.	Sharma et al. 2007

Table 2-3. Continued.

Crops	Target species	Experimental condition	Measurement	Reference
Cotton	*Helicoverpa zea, predatory species*	Abundance	Populations of predators were consistently as high or higher in *Bt* cotton.	Hagerty et al. 2005
Cotton	Pests and non-target arthropods	Abundance	*Bt* cotton has no significant adverse impacts on the nontarget arthropod populations. Also *Bt* cotton supports higher natural enemy populations.	Head et al. 2005
Rice	*Stenchaetothrips biformis*	Abundance	Abundances of *S. biformis* collected from the *Bt* plots were significantly less or same than control plots	Akhtar et al. 2010
Rice	Spider species	Abundance, community structure	No effect	Lee et al. 2014

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Chapter III.

Levels of Cry1Ac1 protein in herbivorous and predatory arthropods in fields of *Bacillus thuringiensis* cabbage

Abstract

To investigate the extent of exposure and routes of Cry1Ac1 protein through the food chain, we collected *Bt* cabbage leaves and arthropods that occurred in the field during two trials in 2014. Protein levels in the transgenic leaves were significantly higher during the early stages of plant growth, ranging from 209.1 to 553.6 ng g^{-1} in Spring and from 208.2 to 402.8 ng g^{-1} in Autumn. Enzyme-linked immunosorbent assays were used to measure protein levels in the arthropods. Among the 16 arthropod taxa collected in the field, Cry1Ac1 was detected in the bodies of 10 taxa. Concentrations were higher in lepidopteran larvae than in the other taxa. In particular, we found a significant correlation between Cry1Ac1 protein levels in cabbage leaves and in *Pieris rapae* and *Mamestra brassicae*. Furthermore, Cry1Ac1 protein was detected in five out of nine taxa of predators (spiders and coleopterans) and parasitoids. These results will be useful as we identify the arthropods that are directly or indirectly exposed to *Bt* toxin within the food web and the degree to which they are exposed during the cultivation of *Bt* cabbage.

Keywords: Arthropods, *Bacillus thuringiensis*, cabbage field, Cry1Ac1 protein, ELISA

3.1. Introduction

Insect resistance is one of the two main traits for GM crops cultivated in the past last two decades (James 2014). This trait is primarily acquired due to expression of the *cry* gene from *Bacillus thuringiensis* (*Bt*). Although *Bt* crops can contribute to increased crop productivity, their potential environmental impacts on non-target organisms have been a major concern (Shelton et al. 2002; Clark et al. 2005). For assessing the non-target effects of these crops, it is essential to identify the species that are exposed to *Bt* protein and assess any correlations between natural and trophic levels of exposure (Harwood et al. 2005; Obrist et al. 2006; Yu et al. 2014). Having a concise database that describes the body concentrations of *Bt* protein in each arthropod species can help researchers select appropriate surrogate species for better understanding the environmental risks of *Bt* crops (Carstens et al. 2013; Romeis et al. 2014).

Under agronomic field conditions, phloem or xylem feeders such as aphids, leafhoppers, and planthoppers may not be exposed to considerable amounts of *Bt* proteins. However, depending upon their feeding styles, some arthropod species can be directly or indirectly exposed to those proteins in transgenic crops. For example, leaf feeders such as lepidopteran caterpillars, leaf beetles, spider mites, and vegetable leaf miners; pollen feeders (e.g., bees, green lacewings, and flower bugs); and plant nectar feeders (parasitoid wasps) (Triplehorn and Johnson 2005)

can be directly exposed (Groot and Dicke 2002; Romeis et al. 2009). In contrast, upper trophic-level organisms, including predators, can be indirectly exposed to *Bt* proteins when they prey upon the herbivores that have consumed *Bt* crops (Groot and Dicke 2002; Romeis et al. 2009). In fact, transmission of *Bt* protein through the food web has been reported from surveys of fields containing *Bt* maize (*Zea mays*), cotton (*Gossypium hirsutum*), soybean (*Glycine max*), and rice (*Oryza sativa*) (Harwood et al. 2005; Obrist et al. 2006; Torres et al. 2006; Zhang et al. 2013; Yu et al. 2014). In such experiments, concentrations of *Bt* protein in arthropod bodies have varied according to the functional group or species, and were noticeably decreased when such proteins were transferred to higher trophic level, such as predators or parasitoids. Those results suggested that no potential for bioaccumulation exists in arthropods.

We have previously described the community structure of arthropods associated with transgenic cabbage (*Brassica oleracea* L. var. *capitata*) plants that express Cry1Ac1 protein (Kim et al. 2015). We have also examined the effect that this protein has on the growth and survival of wolf spiders (*Pardosa astrigera*) (Kim et al. 2016). This cabbage was developed to control damage from diamondback moth (*Plutella xylostella*), a major pest of Brassica crops. As an extension of our earlier works (Kim et al. 2015, 2016), this current study has two objectives. First, we examined the levels of Cry1Ac1 protein in *Bt* cabbage and in

the arthropod species that are exposed to those plants under field conditions. Although the consequences of exposure by arthropods to *Bt* protein have been examined with field-grown *Bt* maize, cotton, and soybean (Harwood et al. 2005; Obrist et al. 2006; Torres et al. 2006; Yu et al. 2014), we are unaware of any investigations with arthropods collected from field-grown *Bt* Brassica crops. Second, we used the exposure levels that we found for *Bt* protein in arthropod species as a tool for screening competent surrogate species in order to conduct non-target risk assessments that reflect realistic conditions in a cabbage field.

3.2. Materials and Methods

3.2.1. Plant materials and field experiments

A transgenic line (C95) of cabbage (*Brassica oleracea* var. *capitata*) was developed from AD126, a non-transgenic control line, to contain *cry1Ac1* (GenBank Accession No. AY126450; Park et al. 2003). Expression is under the control of the cauliflower mosaic virus 35S promoter and the *nos* terminator obtained from a soil bacterium, *Bacillus thuringiensis*. The transgenic line also has *neomycin phosphotransferase II* to impart kanamycin resistance as a selection marker (Harn et al. 2011). Plants of these transgenic and non-transgenic cabbage lines were provided by the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea.

In 2014, we conducted two consecutive trials in the same experimental field at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju, Chungcheongbuk-do, Korea (36°43′N, 127°26′E; elevation, 35 m). The first trial (Spring) ran from May to July. Here, 7-week-old cabbage seedlings that were considered conventionally ready, i.e., having five or six true leaves (Andaloro et al. 1983), were transplanted on 19 May. Both genotypes, C95 and the AD126 control were arranged in a randomized block design with three replications. Each 4 × 4 m plot comprised five planting rows plus black plastic

mulch film to control weeds. The total field area was 180 m^2 (length × width: 18 m × 10 m), and plots were separated by 2 m. In each row, eight seedlings were planted 50 cm apart (40 plants per plot). Pesticides were not sprayed during the entire study period. For the second trial (Autumn), 7-week-old seedlings were transplanted on 29 September, following the same experimental design and practices as for the first trial.

Each week during both trial periods, from seedling to mature plant stage (total of 6 times per trial), we collected arthropods that lived on 120 C95 cabbage plants either by hand or with an aspirator powered by a D-cell battery (Hausherr's Machine Works, Raleigh, NC, USA). The samples were brought to the laboratory and sorted to the species or family level. Concurrently, the leaves of transgenic and non-transgenic lines were sampled. All experimental materials (leaves and arthropods) were stored at –80°C.

3.2.2. ELISA procedure

The concentrations of Cry1Ac1 protein in transgenic and non-transgenic cabbage leaves and the arthropods were determined by double antibody sandwich Enzyme-Linked Immunosorbent Assays (ELISAs), using Cry1Ab/Cry1Ac protein-specific kits (Agdia Inc. USA). Frozen samples of those materials were

first rinsed with deionized water to eliminate any residue of Cry1Ac1 protein on their surfaces (Meissle and Romeis 2009; Yu et al. 2014), and then freeze-dried (Free Zone 2.5; LABCONCO, USA). For small arthropod species, we pooled all of the specimens that were collected from three replicated plots to obtain a sufficient amount for ELISA. The freeze-dried leaves and arthropods were ground to a powder with an auto mill (Tokken, Japan). If the dry weights of those ground samples were lower than 15 μg, they were not further analyzed. All ELISA processes were conducted according to the manufacturer's instructions.

The level of protein in each sample was determined from a seven-point (0, 1, 10, 100, 200, 500, and 1000 (ng g^{-1}) / 3 repetitions) standard curve fitted to the optical density values of a *Bt*-Cry1Ac standard (Biosense, Norway). The Limits of Detection (LOD) for the ELISAs were 2.70 ng (Spring) and 3.14 ng (Autumn) of protein per g dry weight of the arthropod sample.

3.2.3. Statistical analysis

Levels of Cry1Ac1 protein in cabbage plants at different sampling points were examined with one-way ANOVAs, followed by Tukey's HSD tests using STATISTICA v 8.0 (Statsoft, USA). To correlate protein levels in the two lepidopteran herbivore species with levels in the C95 leaves as a function of plant

growth stage, we fitted the linear regressions using STATISTICA v 8.0 (Statsoft, USA).

3.3. Results

3.3.1. Cry1Ac1 protein levels in cabbage plants

The concentrations of Cry1Ac1 protein in transgenic cabbage varied significantly by sampling date during Spring ($F=11.80$, $P<0.001$) and Autumn ($F=4.57$, $P=0.014$). In Spring, levels in *Bt* cabbage ranged from 209.0 to 553.6 ng g^{-1} (Fig 3-1A), with the concentration being significantly greater on 5 June than on any other sampling dates during the first trial (HSD, $P<0.001$). In Autumn, Cry1Ac1 levels in the leaves ranged from 208.1 to 402.4 ng g^{-1} (Fig 3-1B), and were significantly greater on 13 and 20 October than on 17 November. This protein was not detected in leaves from the control cabbage line (AD126) during either trial period.

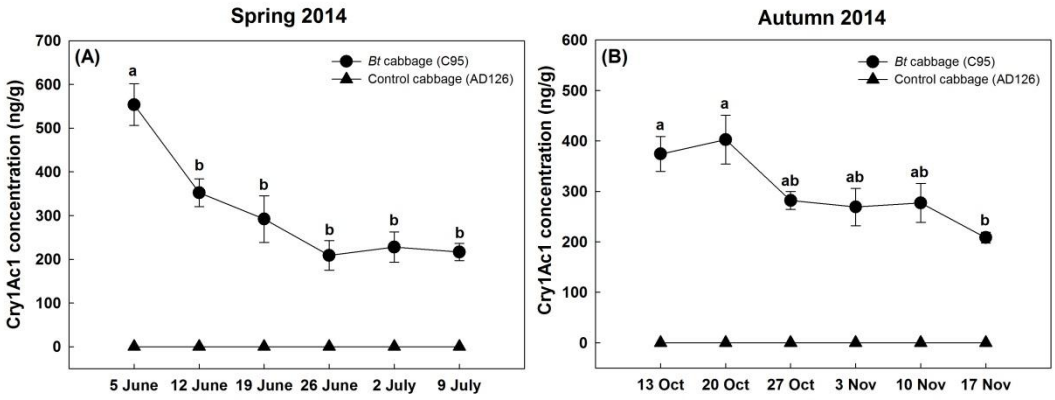

Fig 3-1. Cry1Ac1 protein concentrations in transgenic cabbage and non-transgenic cabbage plants during Spring (A) and Autumn (B) trials in 2014. Data are means (n=3) and standard errors. Values followed by the same letters are not significantly different (Tukey's HSD test, $P<0.05$).

3.3.2. Cry1Ac1 protein levels in arthropod species during two growing seasons

Our collections produced a total of 16 arthropod taxa. Among all 16 taxa, nine were found in both Spring and Autumn, six only in Spring (for a total of 15 taxa overall in that trial), and one only in Autumn (for a total of 10 taxa overall in that trial) (Table 3-1). These samples belonged to 14 families in seven orders. We classified seven taxa as herbivores, eight as predators, and one as belonging to a parasitoid group.

In the case of the herbivores, Cry1Ac1 protein was detected in Lepidoptera, Hemiptera, and Orthoptera species. Among the lepidopteran species, i.e., *Mamestra brassicae*, *Pieris rapae*, *Plutella xylostella*, and *Trichoplusia ni*, protein levels in *M. brassicae* and *P. rapae* followed a pattern similar to that of Cry1Ac1 measured in the transgenic cabbage leaves during both trials (Fig 3-2). For other lepidopteran species, including *P. xylostella* and *T. ni*, for which only one sub-sample could be collected on each sampling date, their protein levels were 5.4 to 19.6% of the concentrations measured in the leaves. The other herbivores, *Eurydema gebleri* (Hemiptera) and *Gryllotalpa orientalis* (Orthoptera), contained only 0.9 to 6.6% of the leaf concentration. No Cry1Ac1 protein was detected in *Nysius plebejus* (Hemiptera), and only one sub-sample was collected for that species at each sampling point.

Protein levels in all predator species were analyzed using only one or two sub-samples per sampling time because of either small body sizes or low abundance (Table 3-1). Among the spider species, those within Erigonidae contained higher amounts of Cry1Ac than species belonging to Araneidae and Thomisidae. Although one sub-sample was used for those spiders, Cry1Ac1 levels ranged from 2.6 to 4.1% of the concentrations measured in the leaves. For the other spiders, including Araneidae and Thomisidae species, Cry1Ac1 levels were mostly less than 1.1% of the leaf concentration or were below the limits of detection. Among coleopteran predators, *Tachyura laetifica* and *Harmonia axyridis* contained 1.2 to 4.6% of the leaf concentrations. However, Cry1Ac1 was not detected in two coleopteran predators (*Propylea japonica* and *Paederus fuscipes*), a neuropteran predator (Chrysopidae sp.), or in the only parasitoid taxa (*Cotesia* sp.) that lived in the field.

Table 3-1. Levels of Cry1Ac1 protein in arthropods collected in cabbage field during 2014 growing season (Spring and Autumn trials). Numbers in parentheses are the number of individuals per subsample.

Order	Family	Species	Functional group	Stage	Mean Cry1Ac1 protein concentration (ng / g DW)					
Spring					5 Jun	12 Jun	19 Jun	26 Jun	3 Jul	10 Jul
Araneae	Araneidae	Araneidae spp.	Predator	Juvenile	n.c.[a]	n.c.	3.06 (1)	n.c.	n.c.	n.c.
	Erigonidae	Erigoninae spp.	Predator	Mix	n.c.	n.c.	9.72 (1)	n.c.	n.c.	n.c.
	Thomisidae	Thomisidae spp.	Predator	Juvenile	5.56 (1)	n.c.	n.c.	−[b] (1)	− (1)	n.c.
Coleoptera	Carabidae	*Tachyura laetifica*	Predator	Adult	n.c.	n.c.	13.06 (1)	n.c.	10.56 (1)	8.06 (1)
	Coccinellidae	*Harmonia axyridis*	Predator	Adult	6.39 (2)	n.c.	n.c.	n.c.	5.14 (2)	n.c.
		Propylea japonica	Predator	Larva	n.c.	n.c.	− (1)	n.c.	n.c.	n.c.
	Staphylinidae	*Paederus fuscipes*	Predator	Adult	n.c.	n.c.	n.c.	n.c.	− (1)	n.c.
Hemiptera	Lygaeidae	*Nysius plebejus*	Herbivore	Adult	n.c.	n.c.	n.c.	n.c.	n.c.	− (1)
	Pentatomidae	*Eurydema gebleri*	Herbivore	Mix	4.82 (3)	n.c.	8.89 (1)	n.c.	15.14 (2)	n.c.
Hymenoptera	Braconidae	*Cotesia* sp.1	Parasitoid	Cocoon	n.c.	n.c.	− (2)	− (1)	n.c.	n.c.
Lepidoptera	Noctuidae	*Mamestra brassicae*	Herbivore	Larva	n.c.	n.c.	57.64 (2)	36.39 (2)	17.22 (3)	25.00
	Pieridae	*Pieris rapae*	Herbivore	Larva	203.61 (3)	72.22 (3)	48.89 (3)	11.81 (2)	n.c.	29.72 (1)
	Plutellidae	*Plutella xylostella*	Herbivore	Larva	n.c.	n.c.	n.c.	n.c.	12.22 (1)	n.c.
Neuroptera	Chrysopidae	Not identified	Predator	Larva	− (1)	− (1)	n.c.	n.c.	n.c.	n.c.
Orthoptera	Gryllotalpidae	*Gryllotalpa orientalis*	Herbivore	Adult	n.c.	n.c.	n.c.	n.c.	n.c.	6.11 (3)

ELISA results not exceeding limit of detection (LOD) were marked as '< corresponding LOD value'.
[a] n.c.: not collected
[b] −: not detected

Table 3-1. Continued

Autumn					13 Oct	20 Oct	27 Oct	3 Nov	10 Nov	17 Nov
Araneae	Araneidae	Araneidae spp.	Predator	Juvenile	n.c.	n.c.	<3.15 (1)	n.c.	n.c.	n.c.
	Erigonidae	Erigoninae spp.	Predator	Mix	n.c.	n.c.	7.27 (1)	10.91 (1)	n.c.	n.c.
	Thomisidae	Thomisidae spp.	Predator	Juvenile	<3.15 (1)	n.c.	n.c.	n.c.	n.c.	n.c.
Coleoptera	Coccinellidae	*Harmonia axyridis*	Predator	Adult	<3.15 (1)	5.46 (1)	n.c.	n.c.	n.c.	n.c.
	Staphylinidae	*Paederus fuscipes*	Predator	Adult	n.c.	− (1)	n.c.	n.c.	n.c.	n.c.
Hemiptera	Lygaeidae	*Nysius plebejus*	Herbivore	Mix	n.c.	n.c.	− (1)	− (1)	− (1)	− (1)
	Pentatomidae	*Eurydema gebleri*	Herbivore	Mix	n.c.	6.36 (1)	4.55 (1)	n.c.	n.c.	n.c.
Lepidoptera	Noctuidae	*Mamestra brassicae*	Herbivore	Larva	66.06 (3)	57.73 (2)	n.c.	30.91 (1)	66.97 (3)	58.18 (3)
		Trichoplusia ni	Herbivore	Larva	n.c.	n.c.	n.c.	n.c.	37.27 (1)	40.71 (1)
	Pieridae	*Pieris rapae*	Herbivore	Larva	78.79 (3)	54.85 (3)	25.15 (3)	18.79 (3)	n.c.	n.c.

ELISA results not exceeding limit of detection (LOD) were marked as '< corresponding LOD value'.

[a] n.c.: not collected

[b] −: not detected

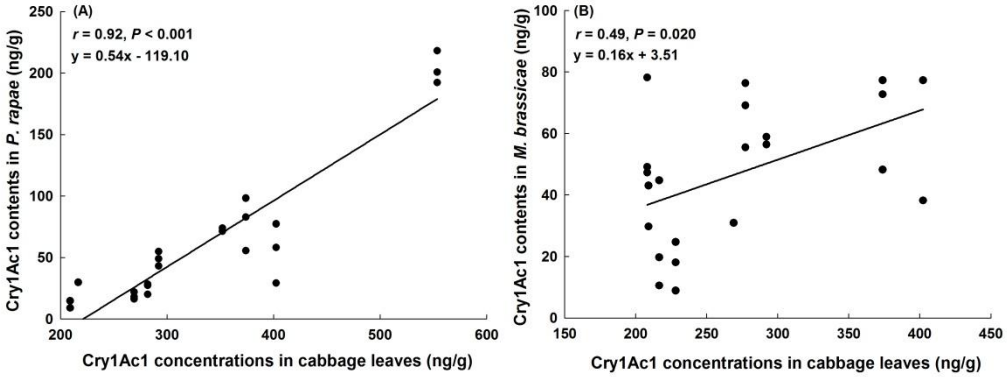

Fig 3-2. Correlation between Cry1Ac1 protein levels in *Bt* cabbage leaves and in 2 lepidopteran herbivore species: (A) *Pieris rapae* and (B) *Mamestra brassicae*.

3.4. Discussion

When assessing the environmental risks of *Bt* transgenic crops using non-target arthropods, it is critical that one selects appropriate surrogate species that reflect the degree of field exposure for the plants being considered (Romeis et al. 2008; Wach et al. 2016). Therefore, it is important to know what concentrations of *Bt* protein are found in arthropods that reside in those transgenic crop fields.

Heteropteran sucking herbivores ingest varying amounts of *Bt* protein when they feed on such crops. For example, under field conditions, leafhopper species on transgenic maize, rice, or soybean contain only low quantities of *Bt* protein (Obrist et al. 2006; Chen et al. 2011; Yu et al. 2014). Likewise, Romeis and Meissle (2011) have indicated that aphid species do not acquire much *Bt* protein in their bodies when they directly feed on transgenic crops. Meissle and Romeis (2009) showed that *Bt* protein levels in aphids can be as low as 0.02% of the *Bt* concentration measured in the maize leaves, or even below LOD values. In contrast, Zhang et al. (2006) and Lawo et al. (2009) have shown that aphids on transgenic cotton ingest a relatively high amount of *Bt* protein, ranging from 12.2 to 25.0% of the leaf concentration. However, Romeis and Meissle (2011) have discounted those results, speculating that this difference was probably caused by contaminations of the aphid samples used for ELISA. In our study, Cry1Ac1 protein in the sap-sucking herbivore *Eurydema gebleri* ranged from 0.9 to 6.6% of

leaf levels during both sampling periods. These values were similar or slightly higher than those reported previously for sucking aphids (<3.0%, Burgio et al. 2007), leafhoppers (<3.5%, Yu et al. 2014), and planthoppers (not detected, Chen et al. 2011). Hori (1968) has shown that the adult cabbage bug *Eurydema rugosa* (Pentatomidae) generally feeds on the plant mesophyll and parenchymatous cells while its nymphs feed on both the mesophyll and the phloem sap. This suggests that, among sucking herbivores, the pentatomid species can ingest more *Bt* protein than do other phloem sap feeders. Our findings are in accord with results by Yu et al. (2014), who demonstrated with *Bt* soybean that the pentatomid species ingest measurable amounts of *Bt* protein, i.e., 1 to 10% of leaf levels. Because we have previously found that the abundance of *E. gebleri* does not differ between *Bt* and non-*Bt* cabbage in the field (Kim et al. 2015), we suggest that transgenic *Bt* cabbage does not have a detrimental effect on that pentatomid species.

The levels of Cry1Ac1 proteins were always higher in our leaf-feeding lepidopteran herbivores than in any other species, with lepidopteran insect concentrations ranging from 5.4 to 36.8% of levels in our *Bt* cabbage leaves. In general, lepidopteran herbivores are primary leaf-eaters that often ingest considerable amounts of *Bt* protein from transgenic crops under either laboratory or field conditions (Dutton et al. 2002; Torres et al. 2006). Torres et al. (2006) have reported that bodies of *Spodoptera eridania* and *Pseudoplusia includens* can

contain 22.2 to 60.0% and 7.4 to 37.5%, respectively, of the amount measured in leaves from field-grown *Bt* (Cry1Ac) cotton. This supports our belief that lepidopteran herbivores are exposed to relatively higher levels of Cry proteins from transgenic *Bt* crops when compared with other herbivorous species.

In our study, we found the transmission of *Bt* protein to natural enemies through tritrophic interactions. We detected *Bt* protein in five arthropod predators from two orders, Araneae and Coleoptera. Among the predator species, spiders are common inhabitants of most terrestrial environments, and they can consume many kinds of arthropods including pest species because they have various ecological behaviors. The spiders collected in our study might have preyed upon stink bug, *E. gebleri* and larvae of *P. rapae*, *P. xylostella* and *M. brassicae* in the cabbage field. The crab spiders (Thomisidae spp) can catch their prey by detecting visual movement (Forster 1979). Sheetweb spiders (Erigonidae species) live at the base of cabbage plants and occur more frequently than other predators in those fields. Accordingly, we measured relatively higher concentrations of Cry1Ac1 protein (2.8 to 4.1% of leaf levels) among this predator group. Yu et al. (2014) have shown that Linyphiidae spiders in soybean fields contain detectable amounts of *Bt* protein. Members of Erigonidae and Linyphiidae species make horizontally oriented webs and live at the base of several crop plants. In China, they are considered one of the major natural enemies in cabbage fields (Sengonca and Liu

2002). Therefore, we suggest that those spiders would be a suitable surrogate species for risk assessments using tritrophic interactions in transgenic cabbage. In the case of coleopteran species, *H. axyridis* contained measureable amouts of *Bt* protein in their body. In general, *H. axyridis* prey on not only aphid species but also infant lepidopteran larvae (Koch 2003). Therefore, *H. axyridis* can be exposed to high amount of *Bt* protein by preying on lepidopteran larvae such as *P. rapae*, *P. xylostella* and *M. brassicae* which consumed *Bt* crops.

The parasitoid *Cotesia* species attacks the larvae of *P. rapae*. However, we did not detect Cry1Ac1 protein in that species. Furthermore, previous studies have found no *Bt* protein in the parasitoid *Cotesia marginiventris* (cocoons or adults) or *Microplitis mediator* (adult), when collected as part of either laboratory or field experiments (Vojtech et al. 2005; Yu et al. 2014). One likely explanation for the absence of *Bt* protein in hymenopteran parasitoids is that excretion is used as a means of detoxification, as demonstrated with the aphid parasitoid *Aphidius ervi* (Couty et al. 2001). However, further examinations are needed to verify this hypothesis.

In summary, our experiments provide initial data on how exposure to Cry1Ac1 protein is reflected by measured concentrations in the bodies of arthropod herbivores, predators, and parasitoids in fields where transgenic cabbage is grown. Based on our results, Erigonidae predator species appears to be

a competent surrogate species that can represent realistic scenarios of field exposure with *Bt* cabbage on non-target organisms. Moreover, we have identified arthropods that are directly or indirectly exposed to *Bt* toxin within the food web, and have shown how their degree of exposure varies over time during the growing season.

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Chapter IV.

Transgenic Cabbage Expressing Cry1Ac1 Does Not Affect the Survival and Growth of the Wolf Spider, *Pardosa astrigera* L. Koch (Araneae: Lycosidae)

Abstract

Both herbivores that consume transgenic crops and their predators can be exposed to insecticidal proteins expressed in those crops. We conducted a tritrophic bioassay to evaluate the ecotoxicological impacts that *Bt* cabbage (*Brassica oleracea* var. *capitata*) expressing Cry1Ac1 protein might have on the wolf spider (*Pardosa astrigera*), a non-target generalist predator. Enzyme-Linked Immunosorbent Assays indicated that protein levels were 4.61 ng g^{-1} dry weight in fruit flies (*Drosophila melanogaster*) fed with the transgenic cabbage and 1.86 ng g^{-1} dry weight in the wolf spiders that preyed upon them. We also compared the life history traits of spiders collected from *Bt* versus non-*Bt* cabbage and found no significant differences in their growth, survival, and developmental rates. Because *Bt* cabbage did not affect the growth of fruit flies, we conclude that any indirect effects that this crop had on the wolf spider were probably not mediated by prey quality. Therefore, exposure to Cry1Ac1 protein when feeding upon prey containing that substance from transgenic cabbage has only a negligible influence on those non-target predatory spiders.

Keywords: cabbage, Cry1Ac1, non-target arthropod, *Pardosa astrigera*, transgenic

4.1. Introduction

The conventional use of transgenic *Bacillus thuringiensis* (*Bt*) crops that express insecticidal δ-endotoxins (Cry proteins) as an anti-pest agent has greatly increased since 1996 (James 2014). Adoption of these *Bt* crops has helped reduce the amount of damage from targeted pests and the cost of insecticide use (Shelton et al. 2002; Clark at al. 2005; Lu et al. 2012). However, the potential influence of insect-resistant transgenic crops on non-target organisms, including primary consumers and predators, has been an important issue when assessing possible environmental risks from those plants. For example, *Bt* toxins can be transferred not only directly to crop-fed herbivores but also indirectly to their predators via trophic pathways (Carpenter 2011). Therefore, risk assessments have been conducted to address concerns about environmental safety and any negative effects of *Bt* crops on the non-target food chain (Naranjo 2009; Devos et al. 2012).

Among the many important predator groups found in agricultural ecosystems, spiders are the most ubiquitous generalist predators, playing an important role in regulating insect pest populations (Nyffeler and Benz 1987). Spiders can be exposed to *Bt* toxin through various ecological routes. For example, they ingest pollen trapped in web silk (Ludy and Lang 2006a) or else feed on pollen-dusted prey (Ludy and Lang 2006b) or herbivores that have consumed *Bt* crops (Chen et al. 2009; Meissle and Romeis 2009; Tian et al. 2010; Tian et al.

2012). Several studies have assessed the effects of those crops on the abundance of spiders in fields (Peterson et al. 2011). However, well-controlled tritrophic bioassays under laboratory conditions are also necessary (Romeis et al. 2006; Romeis et al. 2008). Reports have been mixed about the possible interactions between engineered crops and spider health. For example, transgenic rice that expresses Cry1Ab protein has been examined with such bioassays to determine whether those plants influence species within Lycosidae (*Pardosa pseudoannulata*) and Linyphiidae (*Ummeliata insecticeps*) that prey upon planthoppers (*Nilaparvata lugens*; Homoptera: Delphacidae) and rice leafrollers (*Cnaphalocrocis medinalis*; Lepidoptera: Pyralidae) (Chen et al. 2009; Tian et al. 2010; Tian et al. 2012). Those examinations have revealed that the Cry1Ab and Cry3Bb1 proteins produced in *Bt* crops do not have a direct impact on the survival, development, growth, or fecundity of *P. pseudoannulata* and *Pirata subpiraticus* (also in the Lycosidae) (Chen et al. 2009; Tian et al. 2012) or *U. insecticeps* (Tian et al. 2010). However, Zhou et al. (2014) have found that enzyme activities in *Pardosa pseudoannulata* and *U. insecticeps* are significantly affected when they preyed upon fruit flies fed with Cry1Ab protein. Studies on *Bt* maize have focused on how Cry3Bb1 might affect the non-target web-building spider *Theridion impressum* but have found no negative influence when those spiders either prey upon arthropod species (mixed prey, lacewing, or corn rootworm) or are exposed

to transgenic maize pollen via web re-ingestion (Meissle and Romeis 2009). Research by Ludy and Lang (2006) has shown that the web-building *Araneus diadematus* (Araneae: Araneidae) is affected, but not detrimentally, when it ingests web-trapped pollen from Cry1Ab-expressing maize.

Although several *Bt* plant–herbivore–spider tritrophic bioassays have been performed, the way in which those crops that produce Cry1Ac proteins interact trophically with pests and spiders has not previously been studied in the laboratory. Here, we conducted a tritrophic bioassay of *Bt* cabbage (*Brassica oleraceae* var. *capitata*) that expresses this protein. This cabbage was developed to be resistant to the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae), and the cabbage worm *Pieris rapae* (Lepidoptera: Pieridae) (Kim 2014). Both pests severely damage cabbage productivity and quality (Root 1973; Talekar and Shelton 1993). Our investigation focused on the wolf spider (*Pardosa astrigera* L. Koch). We considered this an appropriate test species because it is the dominant ground-dwelling spider in Korea. Moreover, it is an important natural enemy of *P. xylostella* on both cabbage and oilseed rape (*Brassica napus*) (Quan et al. 2011). As a generalist predator, this spider is widely distributed throughout terrestrial environments, including agricultural lands in Korea, Japan, China, Taiwan, and Russia (Jung and Lee 2011). For our bioassays, we chose one fruit fly species, *Drosophila melanogaster*, because it is a primary consumer of

cabbage and also a prey item for the wolf spider. As saprophytic insects, fruit flies are attracted to any crops, including cabbage, that provide fermenting tissue for their ovipositioning (Capinera 2001). They utilize the fruits, flowers, and decaying materials of other plant parts (Markow and Grady 2008), and have been observed for several years during our field experiments with cabbage (Kim et al. 2015). We have previously confirmed that they are attracted to 7- to 14-day-old decaying cabbage tissues and can successfully reproduce on those tissues under laboratory conditions (26±1°C). They are relatively popular prey for Lycosid spiders in terrestrial environments, e.g., crop fields (Mayntz and Toft 2001; Jespersen and Toft 2003) and can be easily reared in a laboratory (Peng et al. 2013).

4.2. Materials and Methods

4.2.1. Plant materials

Seedlings of transgenic and non-transgenic cabbage lines were provided by the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea. Insecticidal activity in transgenic Line C30 had been verified in the laboratory (Kim 2014) and also observed in the field (Peng et al. 2013). Plants of this line were derived from the inbred, non-transgenic Line AD126 and contained *cry1Ac1* under the control of the cauliflower mosaic virus 35S promoter and the *nos* terminator. Both genotypes were cultivated in an experimental field at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju, Chungcheongbuk-do, Korea (36°43′N, 127°26′E; elevation, 35 m) from April to November 2012. As estimated by Enzyme-Linked Immunosorbent Assays (ELISAs), Cry1Ac1 protein concentrations in these field-grown plants varied between 36 and 125 ng g^{-1} dry weight (DW), depending upon growth stage and sampling date (Fig 4-1). We also measured the levels of Cry1Ac1 protein in leaves from transgenic cabbage that had been decaying at room temperature (RT; 26±1°C) in the laboratory for 7 to 14 d. Protein concentrations were 110, 114, and 80 ng g^{-1} DW after 0, 7, and 14 d, respectively (Fig 4-2).

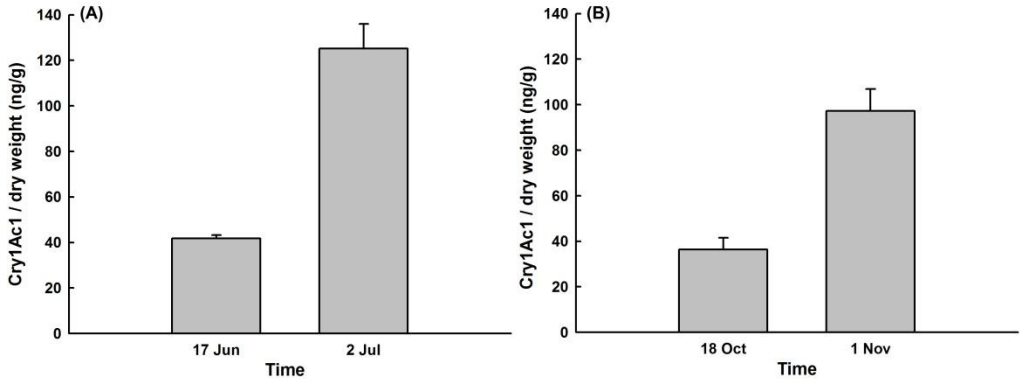

Fig 4-1. Cry1Ac1 protein concentrations in leaves of field-grown *Bt* cabbage plants (Line C30) in Summer 2012 (A) and Autumn 2012 (B). Data are means and standard errors (*n*=3).

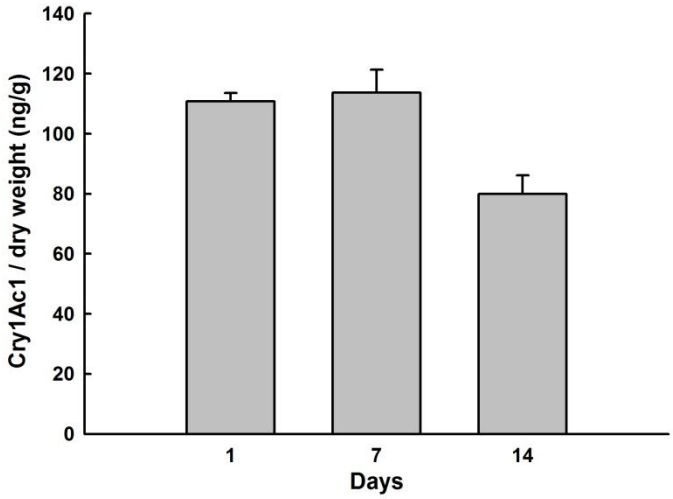

Fig 4-2. Cry1Ac1 concentrations in leaves of *Bt* cabbage (Line C30) decayed for 1, 7, or 14 d in the laboratory. Data are means and standard errors (n=3)

Conventional insecticides and fungicides were not sprayed during the study period. The harvested heads of transgenic and non-transgenic cabbages were placed in a freeze drier (Freezone 2.5; LABCONCO, USA), then ground into powders with a blender (7011HS; Waring, USA) before being stored at –20°C.

4.2.2. Verification of insecticidal activity of *Bt*-drosophila media

To verify the persistence of insecticidal bioactivity in fruit fly-rearing media containing *Bt* cabbage powder, we performed bioassays using larval colonies of the target species, *Plutella xylostella*, that had been obtained from the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea. In preparation, batches of culture media were made from cane sugar (18.0 g), corn meal (20.0 g), wheat meal (3.0 g), dry yeast (5.4 g), and agar (3.6 g) dissolved in 450 mL of distilled water. After the media were boiled and cooled to 50°C, either transgenic or non-transgenic cabbage powder (50 g) was added along with propionic acid (0.75 mL) and 4 mL of nipagin (100 g L-1 methyl 4-hydroxybenzoate in 95% ethanol). Afterward, 15-mL aliquots of these media were transferred to 30-mL drosophila bottles (Hansol Tech, Korea) that were then plugged with sponges. For the bioassays, three media treatment groups were used, based on how much time had elapsed since the powdered cabbage was added (1, 7, or 14 d; all stored at RT). Afterward, all groups were refrigerated at 4±1°C. Samples of the drosophila

media were applied to the cabbage leaves, which were then placed on insect breeding dishes (Cat. No. 310050; SPL Life Sciences Co., Ltd. Korea). Finally, 10 *P. xylostella* larvae (second instar) were added to the dishes and held at RT. The media-treated leaves were replaced with fresh ones every 48 h, and the numbers of live or dead larvae were recorded at 24-h intervals for 7 d.

4.2.2. The quality of *D. melanogaster* as prey

Fruit fly adults, obtained from Hansol Tech, were reared in drosophila bottles at RT, 60±5% relative humidity (RH), and a 16-h photoperiod. As parents, 30 pairs of *D. melanogaster* adults were transferred to sponge-plugged drosophila bottles (30-mL volume; Hansol Tech) that contained media supplemented with transgenic or non-transgenic cabbage powder (five replications each). The insects were maintained for 24 h. As the new generation reached adulthood, the dates were recorded and the mature insects were immediately collected and transferred to a deep freezer (–80±1°C) for 1 h. After each experimental group of adults was separated by gender, their body sizes and weights were recorded. The lengths of the thorax and wing were measured by using a dissecting microscope mounted with a digital camera (excope X3, Korea). To attain a minimum weight level on the digital balance, we measured five individuals together per gender group.

4.2.3. Tritrophic study

Every two weeks, each group of *D. melanogaster* adults was transferred to new media containing either transgenic or non-transgenic cabbage powder. Four replicates of the drosophila media and fruit flies (200 individuals per powder treatment) were freeze-dried for the ELISAs.

To obtain young wolf spiders, we collected 20 adult females carrying egg sacs in the KRIBB experimental field on 2 August 2013. They were kept in sponge-plugged drosophila vials filled with nursery bed soil (10-mL volume, 22 mm in diameter, 92 mm tall; Hansol Tech). The RH was maintained at 60±5% and the vials were kept at RT under a 16-h photoperiod in the insect rearing room. We began with the third instar in tritrophic feeding trials that involved 60 spiders divided into two equal groups. The groups were supplied every 2 d with fruit flies that had fed on either *Bt* cabbage or non-*Bt* cabbage. As the spiders grew larger, the amount of prey was increased from three each in the third and fourth instars to five for the fifth through seventh instars. Feeding experiments were terminated when the spiders reached the adult stage after 74 d.

4.2.4. Evaluation of spider life history traits and body size

The number of surviving spiders was tallied daily. We also determined their developmental times (i.e., how long they spent in each instar), based on the presence of exuvium. When the feeding trials were completed, carapace width (CW), carapace length (CL), and tibia length were measured under a dissecting microscope mounted with an ocular micrometer. The carapace index (CI) was calculated as CW/CL × 100. After fresh weights (FWs) were recorded, four replicates per treatment (six spiders each, with dead ones excluded) were freeze-dried in preparation for the ELISAs. The six spiders were pooled to obtain a sufficiently large sample for this analysis.

4.2.5. ELISA procedure

Using transgenic and non-transgenic cabbage leaves sampled from the field and fermented for 7 and 14 d, we determined the levels of Cry1Ac1 protein in the experimental drosophila media, fruit flies, and wolf spiders via sandwich ELISA, with Cry1Ab/Cry1Ac protein-specific kits (Agdia Inc., USA). Freeze-dried samples of all tissues, both plant and animal, were ground in an auto mill (Tokken, Japan). After their DW values were determined, they were homogenized in 1×phosphate buffer saline-tween wash buffer (PBST, Agdia) at a ratio of 1:10

(w/v). The homogenized samples were centrifuged at 9425 *g* for 5 min before 100 μL of each supernatant was transferred to the test wells of ELISA plates to which the enzyme conjugate and RUB6 diluent mixture were added. The plates were incubated at 25°C for 2 h. Afterward, the primary sample and enzyme conjugate mixture were discarded and the remainder was quickly washed, seven times, with 1×PBST before 100 μL of TMB substrate solution was added to each well. The plates were then incubated for 20 min at RT. Optical density of the test wells was measured on a plate reader at 650 nm. The Cry1Ac1 concentration of each sample was estimated from a seven-point (0, 1, 5, 10, 50, 100 and 200 ng g^{-1}) standard curve fitted to the optical density values of a *Bt*-Cry1Ac standard (Biosense, Norway). The standards were run on each plate of samples and the curves were fitted for each plate.

4.2.6. Statistical analysis

The developmental time and body size of spiders exposed indirectly to either *Bt* cabbage or non-*Bt* cabbage were analyzed with Student's *t*-tests. Sizes and weights of the fruit flies that were directly exposed to *Bt* cabbage or non-*Bt* cabbage were also analyzed with Student's *t*-tests. Survival was assessed by the Kaplan-Meier method and significant differences between the two cabbage groups

were examined by log-rank tests. All statistical analyses were performed using STATISTICA v 8.0 (Statsoft, USA).

4.3. Results

4.3.1. Insecticidal acivity of *Bt* cabbage on target species

Over the 14-d observation period, survival rates for P. xylostella on media containing *Bt* cabbage powder declined relatively rapidly during the first 3 to 4 d, followed by a continuous decrease, such that most of the larvae from all treatment groups were dead by Day 7 (Fig 4-3).

The drosophila medium was stored for 1 (black solid line), 7 (blue dotted line), or 14 (red dashed line) days at room temperature, and was used to verify its insecticidal activity. Data were derived from Kaplan-Meier curves (n=10).

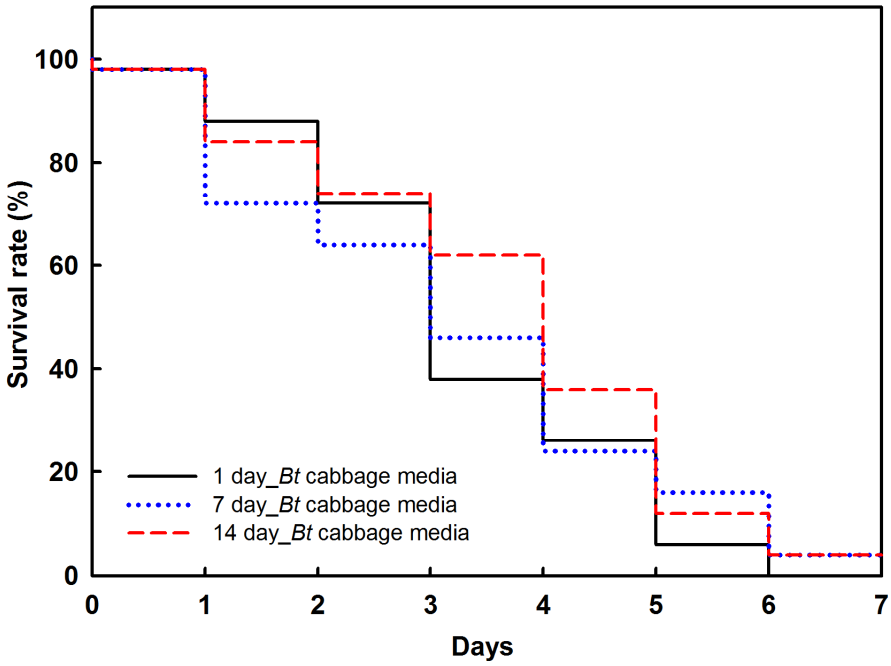

Fig 4-3. Overall survival of *P. xylostella* that had preyed upon drosophila media containing *Bt* cabbage powder.

4.3.2. Effect of *Bt* cabbage on growth of *D. melanogaster*

The new generation of *D. melanogaster* reached adulthood after 9 d for both *Bt* and non-*Bt* cabbage media treatments. Overall, the females had larger body sizes and weights regardless of treatment group, although those differences were not significant (Table 4-1).

Table 4-1. Body size of *D. melanogaster* when exposed to media containing either non-*Bt* cabbage or *Bt* cabbage powder.

Factor	Non-*Bt*	*Bt*	*t*-value	Degrees of freedom	*P*-value[a]
Thorax length, male (mm) (*n*=30)	0.7±0.01	0.7±0.01	1.400	58	0.167
Thorax length, female (mm) (*n*=30)	0.9±0.01	0.9±0.01	0.872	58	0.387
Wing length, male (mm) (*n*=30)	2.0±0.02	2.1±0.02	-1.831	58	0.072
Wing length, female (mm) (*n*=30)	2.4±0.02	2.5±0.03	-0.615	58	0.541
Body weight, male (mg) (*n*=10)	4.1±0.07	4.1±0.05	0.688	18	0.500
Body weight, female (mg) (*n*=10)	6.2±0.1	6.1±0.06	1.044	18	0.310

Data are means±standard errors.
[a]*P*-values are from Student's *t*-tests.

4.3.3. Cry1Ac1 protein concentration

Cry1Ac1 concentrations in *Bt* cabbage leaf powder were 125.2 ng g^{-1} DW; protein levels in the medium containing transgenic-cabbage powder was 44% of the leaf concentration (Fig 4-4). The Cry1Ac1 concentration in fruit fly samples fed with *Bt* cabbage was 10.7% of the amount found on that *Bt* medium while the level in spiders exposed to fruit flies fed with transgenic cabbage was 3.2% of the amount measured from the *Bt* medium. No Cry1Ac1 protein was detected in either the fruit flies that fed on non-*Bt* media or the spiders that preyed upon them.

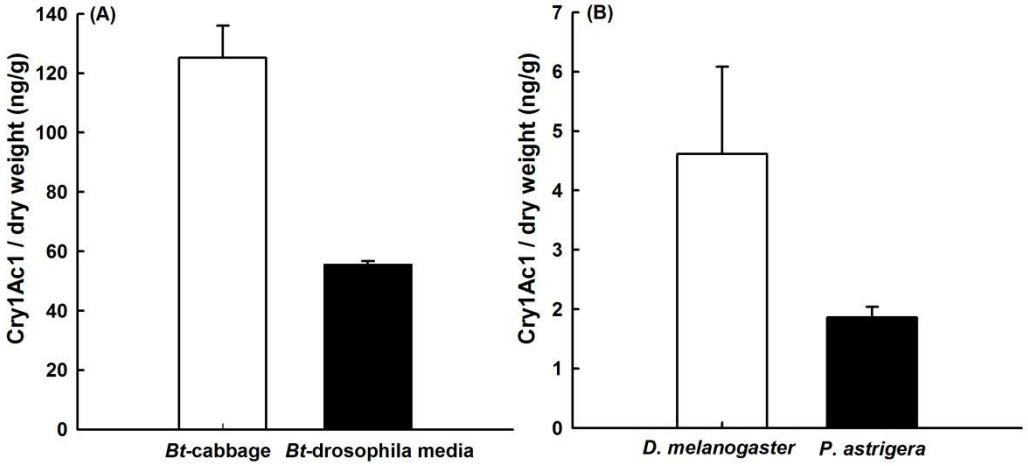

Fig 4-4. Cry1Ac1 concentrations (ng g^{-1} dry weight) in experimental samples. (A) *Bt* cabbage (*n*=3) or drosophila media containing *Bt* cabbage (*n*=4) and (B) *D. melanogaster* feeding on *Bt* cabbage (*n*=4) or *P. astrigera* that had preyed upon *Bt* cabbage-fed *D. melanogaster* (*n*=4). Data are means ± standard errors.

4.3.4. Effects of *Bt* cabbage on spider life history traits and growth

Survival rates for wolf spiders between the third instar and adulthood were not significantly affected by indirect exposure to *Bt* cabbage (Fig 4-5). In addition, preying upon fruit flies fed with transgenic cabbage did not affect the timing of each development stage or the entire life span (Table 4-2).

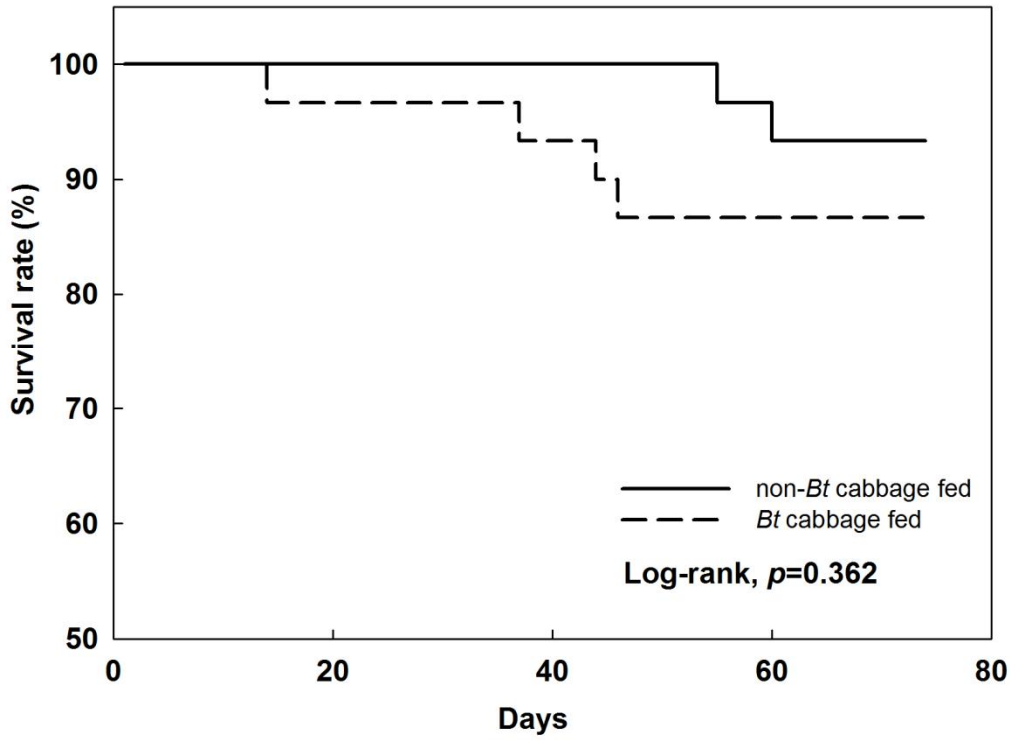

Fig 4-5. Overall survival of the wolf spider, *P. astrigera* that had preyed upon *D. melanogaster* fed with *Bt* or non-*Bt* cabbage.

Data were derived from Kaplan-Meier curves (n=30).

Table 4-2. Time (days) spent in each stage of development for *P. astrigera* from 3rd instar to adult emergence when exposed to either non-*Bt* cabbage-fed or *Bt* cabbage-fed *D. melanogaster*.

Developmental stage	Non-*Bt* (d)	*Bt* (d)	*t*-value	Degrees of freedom	*P*-value[a]
3rd instar	6.2±0.3	6.8±0.3	–1.438	57	0.156
4th instar	11.5±0.6	10.7±0.4	0.831	57	0.409
5th instar	11.9±0.4	12.1±0.5	–0.309	57	0.758
6th instar	16.3±0.7	15.0±0.7	1.219	55	0.228
7th instar	18.5±1.1	16.8±0.6	1.199	15	0.248
3rd instar to adult	52.1±1.0	50.1±1.2	1.589	54	0.118

Data are means±standard errors.
[a]*P*-values are from Student's *t*-tests.

Overall, morphological traits (i.e., FW, carapace width and length, carapace index, and tibia length) were not significantly different among feeding groups (Table 4-3, $P>0.05$).

Table 4-3. Comparisons of body sizes for *P. astrigera* exposed to non-*Bt* cabbage-fed versus *Bt* cabbage-fed *D. melanogaster*.

Factor	Non-*Bt* (*n*=28)	*Bt* (*n*=26)	*t*-value	Degrees of freedom	*P*-value[b]
Fresh weight (mg)	23.8±0.6	24.5±0.8	–0.677	52	0.501
Carapace width (mm)	2.2±0.02	2.2±0.03	1.136	52	0.207
Carapace length (mm)	2.9±0.03	2.8±0.1	0.874	52	0.356
CI[a]	77.0±0.5	77.7±1.4	0.218	52	0.634
Tibia length (mm)	2.4±0.04	2.3±0.1	0.696	52	0.490

Data are means±standard errors.
[a]CI, carapace index was calculated as CI = CW/CL × 100
[b]*P*-values are from Student's *t*-tests.

4.4. Discussion

4.4.1. Biotransfer of *Bt* protein via tritrophic interaction

In our study, the mean concentration of Cry1Ac1 protein in the fruit fly media was 56 ng g^{-1} DW, which was within the range of 36 to 125 ng g^{-1} DW measured from field-grown transgenic cabbage. Therefore, those media concentrations realistically represented what fruit flies are exposed to under field conditions.

After our 74-d feeding trials, we detected Cry1Ac1 protein in both the fruit fly and wolf spider samples, thereby demonstrating that the protein could be transferred from primary consumers to predators through the food chain. Such transfers via tritrophic interactions have also been described previously. For example, Zhou et al. (2014), reported that the bio-transfer rates of Cry1Ab were 5% (5 ng mg^{-1} FW) for the fruit fly primary consumer and 18% (18 ng mg^{-1} FW) for the *P. pseudoannulata* predator when exposed either directly or indirectly to drosophila media containing 100 ng FW mL^{-1} of protein. All of these results suggested that Cry1Ab and Cry1Ac1 proteins do not accumulate in spider predators. Even though Cry protein levels in those food sources and the lengths of the test periods differed among these studies, the amounts of protein measured in the primary consumer and predators followed similar trends. Finally, Meissle and Romeis (2012) have reported that, under both short-term (1-8 d) or long-term

(28-64 d) feeding, bioaccumulations of Cry3Bb1 protein do not occur in the non-target spider *Phylloneta impressa* (Araneae: Theridiidae) or in its prey *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) and *Chrysoperla carnea* (Neuroptera: Chrysopidae).

4.4.2. The effect of *Bt* crops on life history traits of spider predators

To date, *Bt* crops have not been shown to influence negatively the survival and growth of non-target spiders. The same was noted in our examination with *Bt* cabbage and wolf spiders. The morphological traits (body weight, carapace length and width, tibia length) were not significantly different between test groups in the present study. Among those traits, the carapace width has, in particular, been considered an important indicator of the growth of lycosid spiders (Miyaista 1968; Hagstrum 1971; Pickavace 2001).

Similar results have been described for web-building *Theridion impressum* and Cry3Bb1-expressing maize pollen under laboratory conditions (Meissle and Romeis 2009), as well as the garden spider *Araneus diadematus* and Cry1Ab maize pollen (Ludy and Lang 2006). In the case of ground-dwelling spiders, Tian et al. (2012) have observed that the survival, development, and fecundity of *Pardosa pseudoannulata* are not significantly influenced when they prey upon planthoppers that were fed with Cry1Ab protein-expressing rice. Similarly, Chen

et al. (2009) have shown that the survival and fecundity of *Pirata subpiraticus* were not negatively affected when they preyed upon leafrollers fed with *Bt* (Cry1Ab) rice. Although they did find that developmental times were delayed for spiders indirectly exposed to the transgenic rice when compared with the control group, this may have been due to other reasons, such as the nutritional quality of the prey. In contrast, our study results demonstrated that *Bt* cabbage did not affect the growth of fruit flies, thereby making it unlikely that prey quality influenced the life history of these wolf spiders.

4.5. Conclusions

Although numerous field studies have investigated how transgenic cotton and rice producing Cry1Ac proteins affect non-target arthropod communities (Liu et al. 2003; Naranjo 2005; Torres and Ruberson 2005; Han et al. 2014; Lee et al. 2014), less attention has been focused on measuring *Bt* protein concentrations and examining trophically the consequences of *Bt* protein consumption (Wei et al. 2008; Torres and Ruberson 2007). Our tritrophic assay showed that young wolf spiders grew normally from the third instar to adulthood when reared on fruit flies that had been fed either *Bt* cabbage or non-*Bt* cabbage. Although we detected the presence of Cry1Ac1 protein in the spider bodies, it did not affect their

development and survival. Thus, cultivation of transgenic *Bt* cabbage does not influence the survival and growth of wolf spiders.

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Chapter V.

Effects of transgenic cabbage expressing Cry1Ac1 protein on target and the non-target arthropod community under field conditions

Abstract

Under field conditions, we investigated how transgenic *Bt* cabbage expressing the insecticidal Cry1Ac1 protein affects two target Lepidoptera species — *Plutella xylostella* (Plutellidae) and *Pieris rapae* (Pieridae) — as well as the structure of the local non-target arthropod community. When exposed to *Bt* cabbage Line C30, both species were significantly less abundant than when in the presence of the non-transgenic control. Transgenic Line C24 had no apparent influence on those target populations. Multivariate analyses (PerMANOVA and NMDS) showed that composition of the non-target community was affected by sampling date but not by cabbage genotype. These results suggest that transgenic cabbage expressing Cry1Ac1 protein can be effective in controlling *Plutella xylostella* and *Pieris rapae* in the field and that its cultivation does not adversely affect non-target arthropods.

Keywords: cabbage, non-target organism, *Pieris rapae*, *Plutella xylostella*, transgenic crop

5.1. Introduction

Since 1996, transgenic crops expressing insecticidal protein originating from *Bacillus thuringiensis* (*Bt*) have been commercially available for agricultural pest management (James, 2013). Farmers have adopted the use of these insect-resistant *Bt* crops because this practice reduces the need for chemical pesticides while also increasing crop quality and yields (Betz et al., 2000; Clark et al., 2005). However, concerns remain about the potential adverse effects of such crops on non-target arthropods. For example, *Bt* maize pollen and detritus have had detrimental impacts on the survival and growth of the non-target lepidopteran *Danaus plexippus* (Hansen Jesse and Obrycki, 2000; Mattila et al., 2005) and the non-target leaf-shredding trichopteran *Lepidostoma liba* (Chambers et al., 2010).

Cabbage (*Brassica oleracea* L. var. *capitata*) is an economically important crop widely attacked by many insect pests. In particular, leaf-feeding by the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) and the small white, *Pieris rapae* (Lepidoptera: Pieridae) can greatly reduce productivity and quality (Chen et al., 2008a). Although farmers usually apply conventional insecticides to control those pests in cabbage fields, their use can account for 25 to 30% of the total production cost (Dadang and Djoko, 2009).

Several *Brassica* crops, including cabbage (Metz et al., 1995; Yi et al., 2013), oilseed rape (*B. napus*; Stewart et al., 1996), broccoli (*B. oleracea* var.

italica; Zhao et al., 2000; Cao et al., 2002), and collard (*B. oleracea* var. *acephala*; Cao et al., 2005) have been transformed to express *Bt* proteins for controlling lepidopteran pests. Ramachandran *et al*. (1998) showed that transgenic canola expressing Cry1Ac protein effectively controlled its target pest *Plutella xylostella* in the field. The impacts of transgenic *Brassica* crops on non-target organisms such as herbivores (Howald et al., 2003), predators (Tian et al., 2013), and parasitoids (Schuler et al., 2004; Chen et al., 2008b) have also been studied in the laboratory. However, relatively few studies have examined target and non-target species at the population or community level under field conditions (Lang and Otto, 2010).

In the present study, we asked two questions: 1) how effective is *Bt* cabbage in controlling *Plutella xylostella* and *Pieris rapae* in the field? and 2) does *Bt* cabbage expressing Cry1Ac1 protein influence non-target arthropods? To answer these, we compared the abundance of both pests and the community structures of non-target arthropods in the presence of transgenic *Bt* and non-transgenic cabbage.

5.2. Materials and Methods

5.2.1. Plant materials and field experiments

Seedlings of transgenic and non-transgenic cabbage lines were provided by the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea. Two transgenic lines of *Bt* cabbage (C24 and C30), derived from inbred, non-transgenic Line AD126, carry *cry1Ac1*gene (GeneBank accession no. AY126450, Park et al., 2003) under the control of the cauliflower mosaic virus *35S* promoter and the *nos* terminator selection. The transgenic lines also contain the neomycin phosphotransferase II gene (*nptII*) for kanamycin (Harn et al., 2011).

All studies from 2011 to 2013 were conducted in the same experimental field at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon-gun, Chungcheongbuk-do, Korea (36°43´N, 127°26´E; elevation, 35 m). The first trials ran from May to July of 2011. Cabbage is usually ready for transplanting when seedlings have five or six true leaves (Andaloro et al., 1983). Here, seven-week-old plants were placed in the field on 27 May 2011 and mature cabbages were harvested on 15 July 2011. The three genotypes (C24, C30, and the AD126 control) were arranged in a 3 × 3 Latin square design (3 replicated plots per cabbage line). Each 6 × 6 m plot had six planting rows mulched with black plastic film to control weeds. In each row, 12 seedlings were planted 50 cm apart

(72 plants per plot). Each plot was separated by 1 m. Conventional insecticides and fungicides were not sprayed during the study period. For the second trial, seven-week-old seedlings were transplanted on 7 September 2012. Harvesting occurred on 9 November 2012. The same experimental design and practices were followed in both years.

Because plants from Line C24 did not reduce the number of target pests as effectively as those from C30 during either 2011 or 2012, we focused only on the impact of Line C30 for two additional studies conducted in 2013. The first involved seven-week-old seedlings from Lines C30 and AD126 transplanted on 25 April. Mature cabbages were harvested on 2 July 2013. For this, a randomized block design with five replicate plots was adopted. Plot size, number of rows, number of plants per plot, and distance between plots were the same as in 2011 and 2012. In the final trial, seedlings were transplanted on 30 September 2013, followed by harvesting on 22 November. The experimental design was the same as in Spring 2013. Again, conventional insecticides and fungicides were not sprayed during the study periods.

5.2.2. Monitoring of arthropod community

We measured the population densities of our two target pest species as well as non-target arthropods on or near plants from the three lines. For direct counts,

three plants were chosen within the center four rows in each replicated plot during Spring 2011, Autumn 2012, and Spring and Autumn 2013. Their aboveground parts were examined weekly, with tallies of insects recorded six times per trial between 3 June and 8 July in 2011, 27 September and 2 November in 2012, 23 May and 28 June in 2013, and 11 October and 15 November in 2013. The counted insects were not removed from the plants.

Arthropod samples were collected from two yellow sticky traps (250 mm × 150 mm) positioned 30 cm above the second and fourth rows of each plot. Traps were replaced weekly from 12 October to 2 November 2012, 23 May to 28 June 2013, and 11 October to 15 November 2013. They were stored in a freezer (–20°C) until all sampled arthropods were identified.

5.2.3. Statistical analysis

Multiple observations per plot were averaged and the plot mean was used in statistical analyses. Repeated measures ANOVAs (RMANOVAs) were employed to test for significant differences in abundance of the target species between *Bt* and non-*Bt* control plants. Normality and homogeneity of the data were evaluated by Shapiro-Wilk W tests and Levene's tests, respectively. Data were $\log_{10}$ (x+1)-transformed before analysis to satisfy the assumption of normality.

In a separate investigation, we excluded the abundance data for the two targets from the dataset and applied multivariate analysis to examine the effects of *Bt* cabbage on the non-target arthropod community. Because genus or species could not be identified for some arthropods, abundance data at the family level were 4th root-transformed and Bray–Curtis similarity was calculated (Faith et al., 1987). Nonmetric multidimensional scaling (NMDS) (Kruskal, 1964) was conducted to visualize the influence of *Bt* cabbage on the community structure. An NMDS ordination was generated using 100 random restarts with the first Kruskal fit scheme. A two-way permutational multivariate analysis of variance (PerMANOVA; Anderson et al., 2008) was used to test for the significance of differences between non-target arthropod communities based on plant genotype (C24, C30, or AD126 in 2011 and 2012; C30 or AD126 in 2013) and across sampling dates. Tests were performed with type III sums of squares and 9999 permutations, using residuals under a reduced model. Pair-wise post-hoc tests were also applied to compare the levels of each factor.

RMANOVA was conducted with STATISTICA v 8.0 (Statsoft, USA), and NMDS and PerMANOVA were performed using Primer v 6.1.13 with PerMANOVA+ add-on v 1.0.3 (PRIMER-E, UK).

5.3. Results

5.3.1. *Effects of Bt cabbage on Plutella xylostella*

In Spring 2011, genotype significantly affected the abundance of *P. xylostella* (Fig 5-1A; Table 5-1). Tukey's HSD tests showed that counts were significantly lower on plants of C30 than on AD126. However, differences in tallies were not significant between C24 and AD126. Abundance also varied by sampling date, with the population being largest on 1 July among individuals evaluated between 3 June and 8 July. Genotype also affected abundance of this species in Autumn 2012 (Fig 5-1B; Table 5-1). Significantly more insects were found on AD126 than on either C24 or C30. Although abundance was higher on 25 October and 2 November, the overall effect of sampling date was not statistically significant. Abundance on the two *Bt* lines was not greatly changed over time. In Spring and Autumn 2013, significantly fewer insects were found on C30 than on AD126. Sampling date also significantly affected abundance that year (Fig 5-1C, D; Table 5-1). Sticky trap catches of adults in 2012 did not differ among genotypes or sampling dates (Fig 5-2A; Table 5-1). However, in 2013, insects were significantly more abundant from traps placed near AD126 than from those close to C30 (Fig 5-2B, C; Table 5-1).

Table 5-1. Results from a repeated-measures analysis of variance (RMANOVA) for the abundance of *Plutella xylostella* and *Pieris rapae*. Because individuals of *P. rapae* were found on only a few sticky traps in Autumn 2012 and in Spring 2013, we were unable to perform a statistical analysis for that period.

	Spring 2011			Autumn 2012			Spring 2013			Autumn 2013		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
(a) Abundance of *Plutella xylostella* assessed by visual counts												
Genotype (G)	2	6.66	0.029	2	15.17	0.005	1	81.15	0.001	1	26.06	0.001
Time (T)	5	17.08	0.001	5	2.23	0.077	5	35.33	0.001	5	5.96	0.001
G × T	10	3.20	0.007	10	2.96	0.010	5	18.73	0.001	5	6.06	0.001
Error	30			30			140			140		
(b) Abundance of *Plutella xylostella* assessed by sticky trap sampling												
Genotype (G)				2	2.01	0.215	1	5.36	0.033	1	10.81	0.004
Time (T)				3	1.69	0.205	5	16.90	0.001	5	6.16	0.001
G × T				6	0.64	0.698	5	1.84	0.112	5	1.72	0.138
Error				18			90			90		
(c) Abundance of *Pieris rapae* assessed by visual counts												
Genotype (G)	2	1.81	0.242	2	5.63	0.042	1	54.03	0.001	1	11.06	0.002
Time (T)	5	31.11	0.001	5	7.16	0.001	5	50.70	0.001	5	6.35	0.001
G × T	10	2.24	0.043	10	3.39	0.005	5	2.27	0.051	5	2.59	0.028
Error	30			30			140			140		
(d) Abundance of *Pieris rapae* assessed by sticky trap sampling												
Genotype (G)										1	0.51	0.483
Time (T)										5	1.23	0.301
G × T										5	0.10	0.991
Error										90		

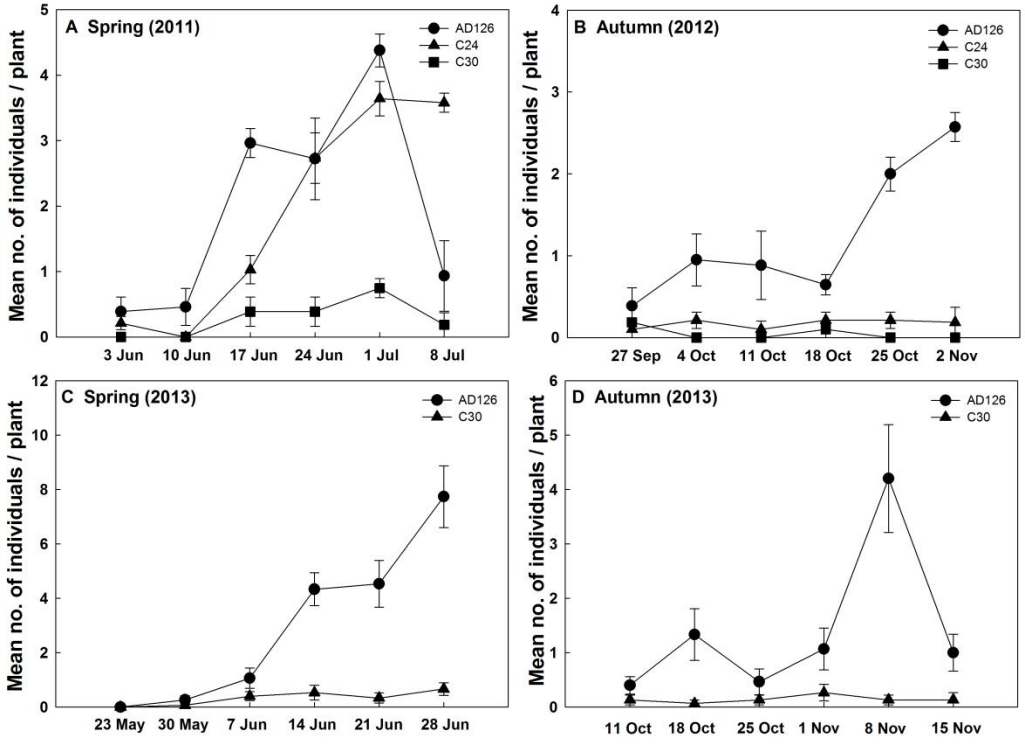

Fig. 5-1. Abundance (mean number of individuals per plant) of *Plutella xylostella* in cabbage field assessed by visual counts in (A) Spring 2011, (B) Autumn 2012, (C) Spring 2013, and (D) Autumn 2013. Bars represent one standard error of mean.

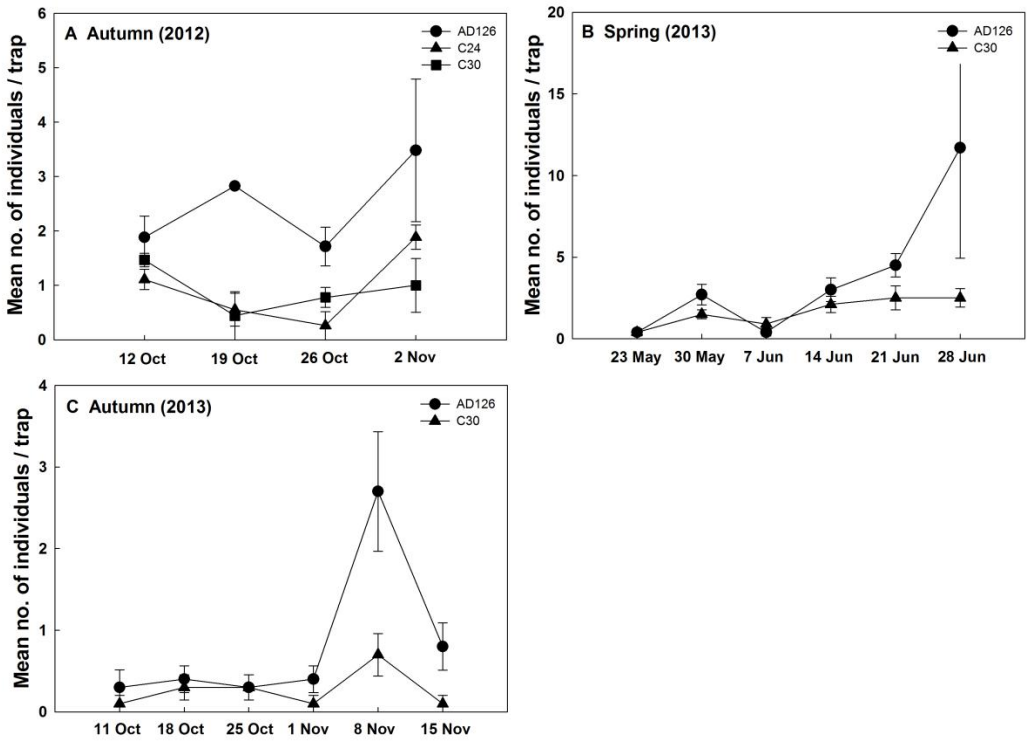

Fig 5-2. Abundance (mean number of individuals per plant) of *Plutella xylostella* in cabbage field assessed by sticky-trap sampling in (A) Autumn 2012, (B) Spring 2013, and (C) Autumn 2013. Bars represent one standard error of mean.

5.3.2. Effects of *Bt* cabbage on *Pieris rapae*

In Spring 2011, cabbage genotype did not significantly affect the abundance of *P. rapae* (Fig. 5-3A; Table 5-1). However, sampling date did have a significant impact, with overall abundance being greatest on 1 July but rapidly decreasing by 8 July. By contrast, genotype influenced abundance in Autumn 2012 (Fig. 5-3B; Table 5-1). Tukey's HSD tests showed that insect counts were significantly lower on C30 than on AD126, but C24 and AD126 did not differ significantly. Time was also an important factor in Spring and Autumn 2013, when abundance was significantly lower on C30 than on AD126 and also was significantly affected by sampling date (Fig. 5-3C, D; Table 5-1). Because individuals of *P. rapae* were found on only a few sticky traps in Autumn 2012 and in Spring 2013, the standard errors of means were great and we were unable to perform a statistical analysis for that period (Fig. 5-4, Table 5-1). In Autumn 2013, neither genotype nor sampling date significantly affected the abundance of trapped *P. rapae*.

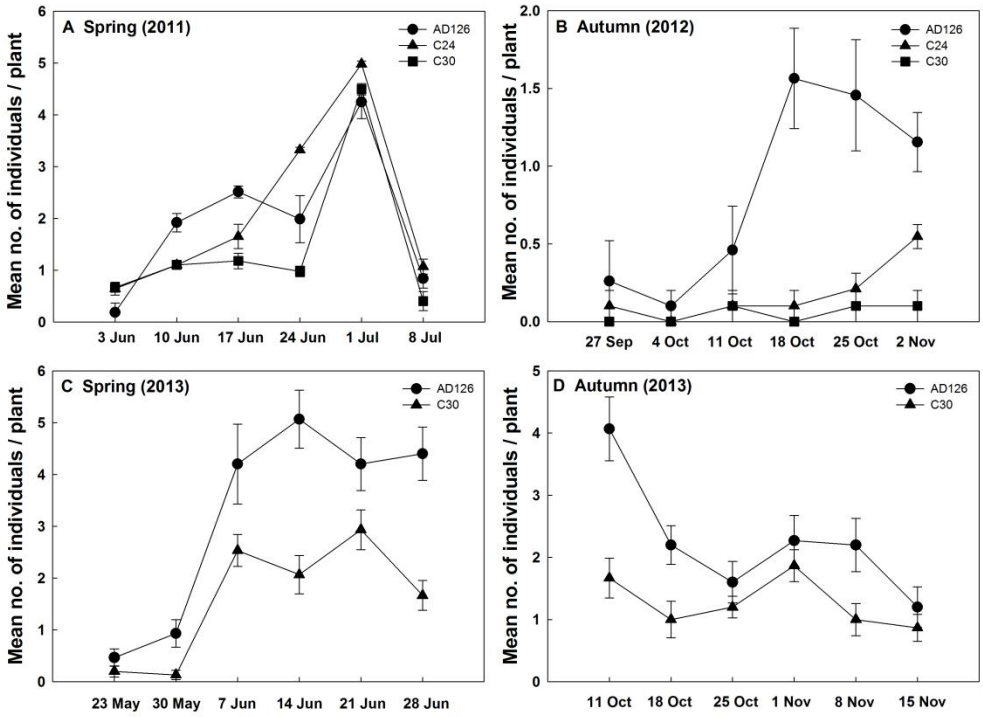

Fig. 5-3. Abundance (mean number of individuals per plant) of *Pieris rapae* in cabbage field assessed by visual counts in (A) Spring 2011, (B) Autumn 2012, (C) Spring 2013, and (D) Autumn 2013. Bars represent one standard error of mean.

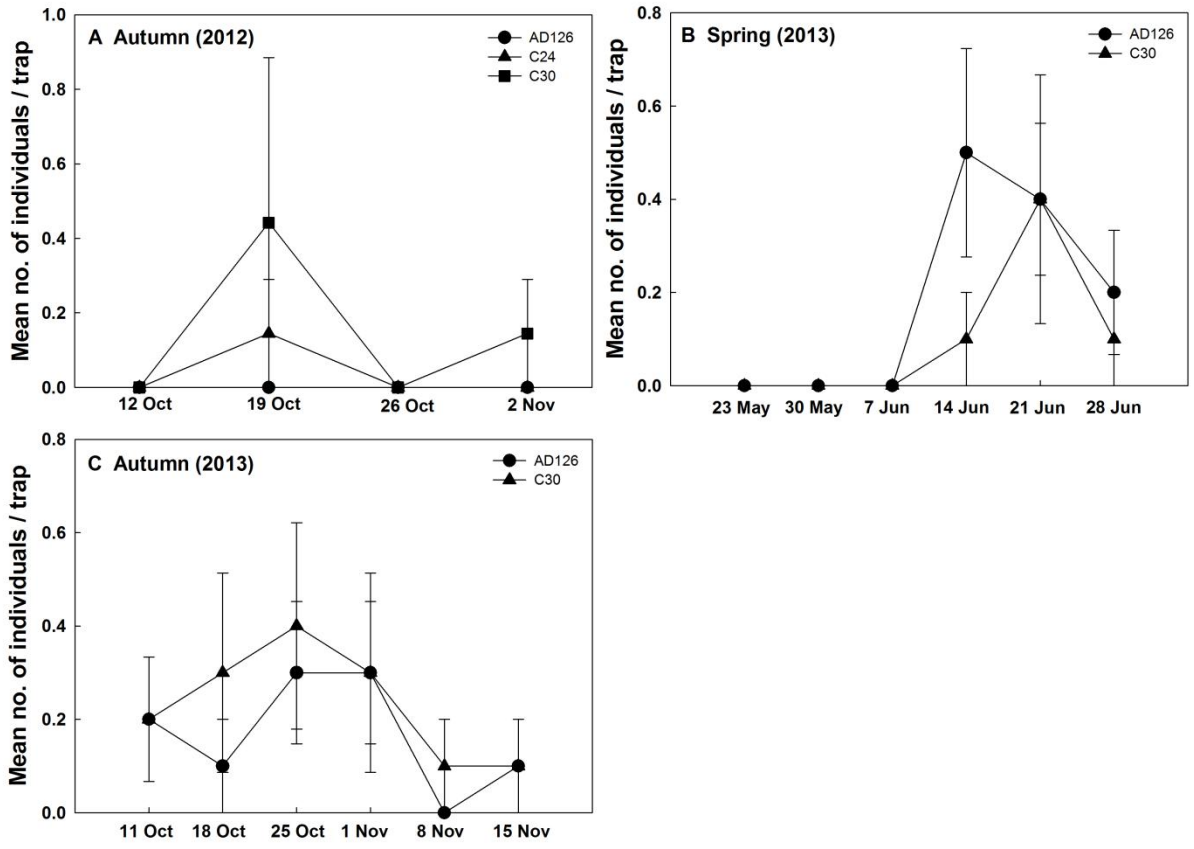

Fig. 5-4. Abundance (mean number of individuals per plant) of *Pieris rapae* in cabbage field assessed by sticky trap sampling in (A) Autumn 2012, (B) Spring 2013, and (C) Autumn 2013. Bars represent one standard error of mean.

5.3.3. Effects of *Bt* cabbage on the non-target arthropod community

When the composition of non-target arthropods was monitored visually and with sticky traps in Spring 2011, Autumn 2012, and Spring and Autumn 2013, cabbage genotype had no influence (Table 2). However, sampling date did have a significant effect. Our PerMANOVA results were consistent with those obtained from NMDS ordinations. There, temporal variations were revealed in the non-target arthropod community, but community structure did not differ among genotypes (Figs 5-6).

Table 5-2. Results from a two-way permutational multivariate analysis of variance (PerMANOVA) for the composition of non-target arthropods based on Bray-Curtis similarity coefficients.

	Spring 2011			Autumn 2012			Spring 2013			Autumn 2013		
	d.f.	Pseudo-*F*	*P*	d.f.	Pseudo-*F*	*P*	d.f.	Pseudo-*F*	*P*	d.f.	Pseudo-*F*	*P*
(a) Abundance assessed by visual counts												
Genotype (G)	2	0.48	0.722	2	0.88	0.518	1	2.08	0.130	1	1.34	0.297
Time (T)	5	9.03	0.001	5	13.10	0.001	5	11.80	0.001	5	8.32	0.001
G × T	10	0.76	0.740	10	0.69	0.768	5	0.79	0.666	5	0.65	0.776
Res	36			36			24			24		
(b) Abundance assessed by sticky trap sampling												
Genotype (G)				2	1.44	0.087	1	0.58	0.821	1	1.42	0.185
Time (T)				3	5.76	0.001	5	18.03	0.001	5	16.53	0.001
G × T				6	0.99	0.510	5	0.55	0.989	5	1.38	0.057
Res				24			108			108		

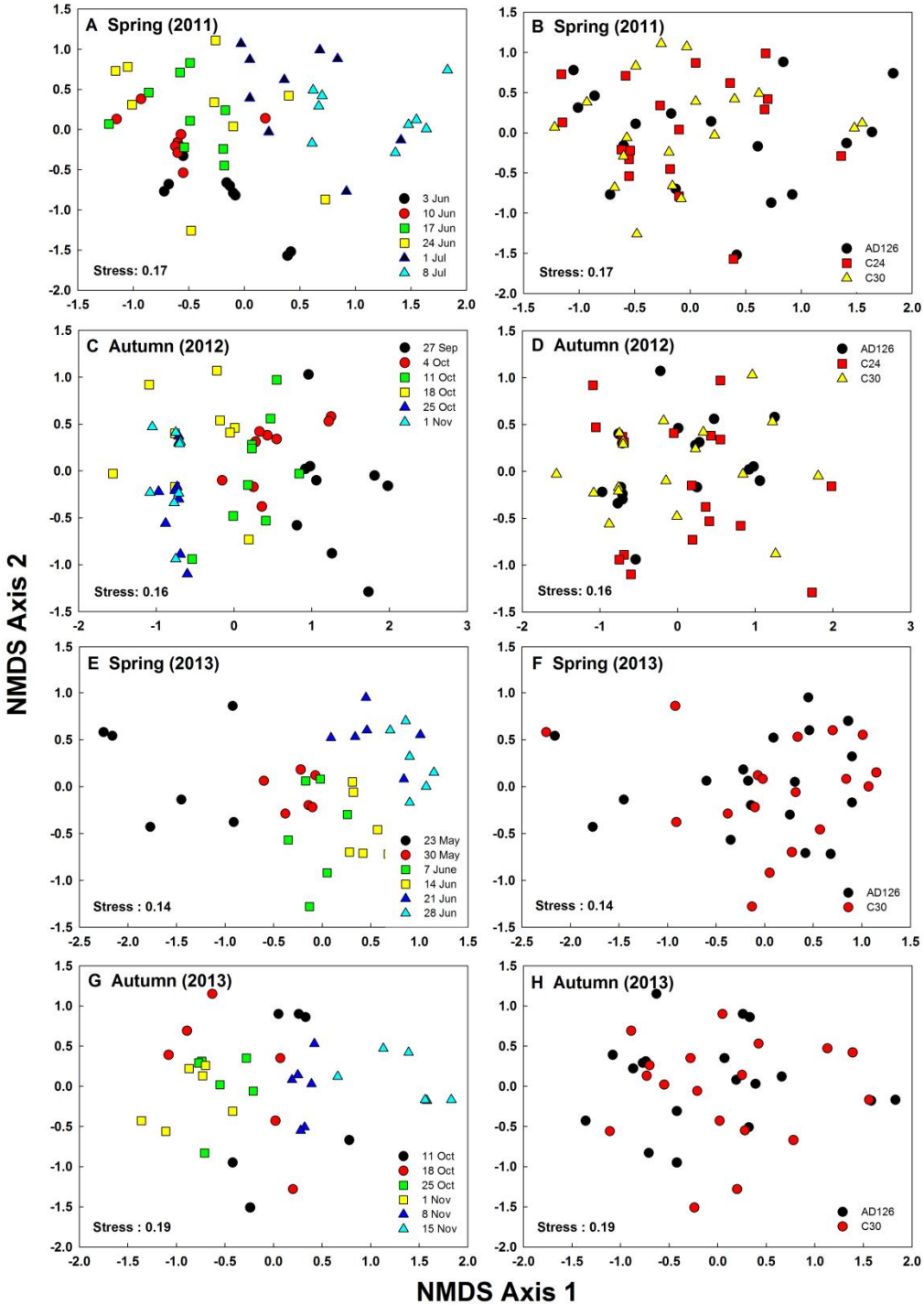
A Spring (2011)
Stress: 0.17
3 Jun
10 Jun
17 Jun
24 Jun
1 Jul
8 Jul
B Spring (2011)
Stress: 0.17
AD126
C24
C30
C Autumn (2012)
Stress: 0.16
27 Sep
4 Oct
11 Oct
18 Oct
25 Oct
1 Nov
D Autumn (2012)
Stress: 0.16
AD126
C24
C30
E Spring (2013)
Stress : 0.14
23 May
30 May
7 June
14 Jun
21 Jun
28 Jun
F Spring (2013)
Stress : 0.14
AD126
C30
G Autumn (2013)
Stress : 0.19
11 Oct
18 Oct
25 Oct
1 Nov
8 Nov
15 Nov
H Autumn (2013)
Stress : 0.19
AD126
C30
NMDS Axis 2
NMDS Axis 1

Fig. 5-5. Non-metric multi-dimensional scaling (NMDS) plots of non-target species in arthropod community assessed by visual counts in Spring 2011 (A and B), Autumn 2012 (C and D), Spring 2013 (E and F), and Autumn 2013 (G and H). Each symbol is a two-dimensional representation of non-target arthropod community. The distances between symbols were calculated by Bray-Curtis dissimilarity coefficient.

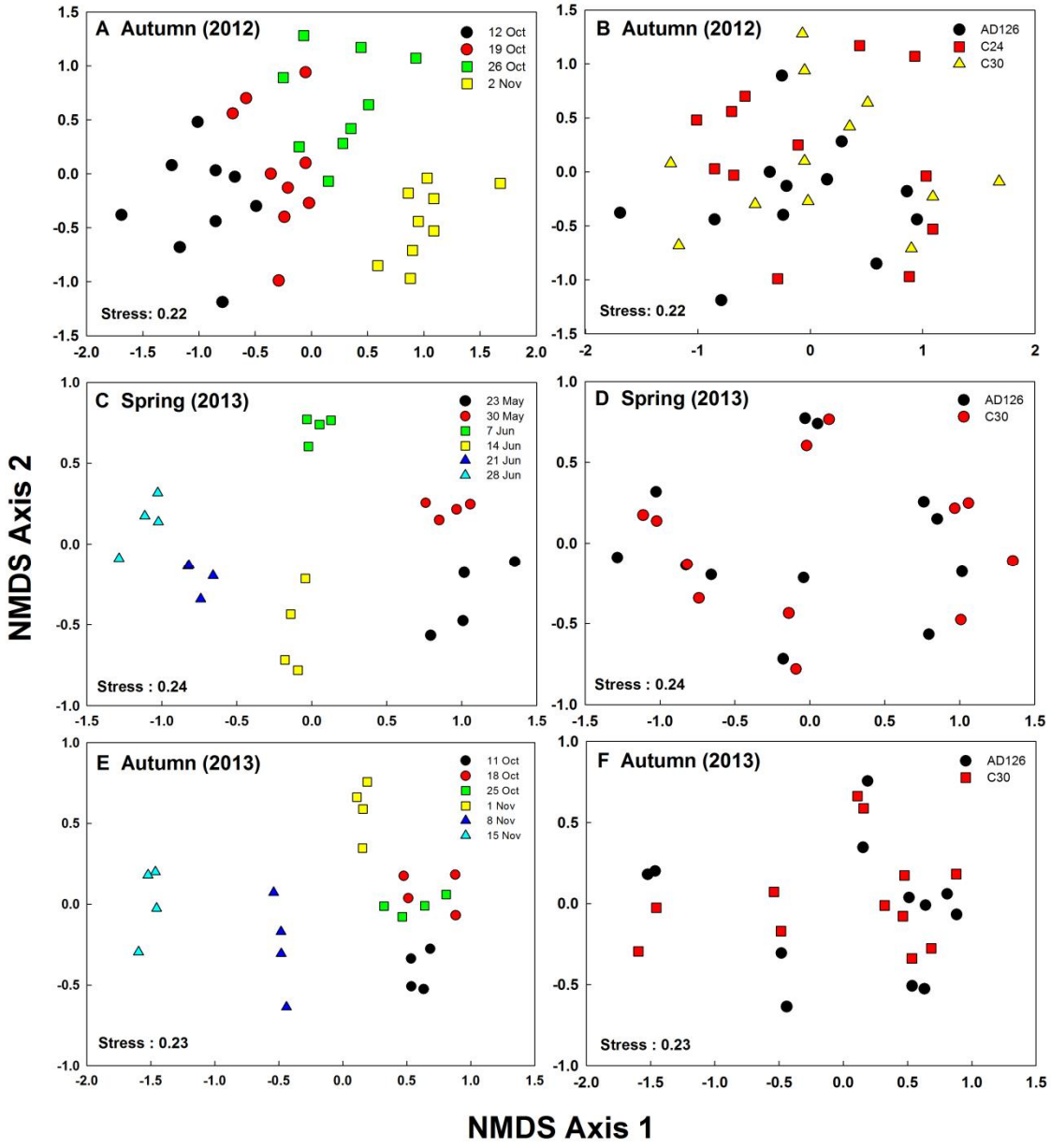
A Autumn (2012)
12 Oct
19 Oct
26 Oct
2 Nov
Stress: 0.22
B Autumn (2012)
AD126
C24
C30
Stress: 0.22
C Spring (2013)
23 May
30 May
7 Jun
14 Jun
21 Jun
28 Jun
Stress : 0.24
D Spring (2013)
AD126
C30
Stress : 0.24
E Autumn (2013)
11 Oct
18 Oct
25 Oct
1 Nov
8 Nov
15 Nov
Stress : 0.23
F Autumn (2013)
AD126
C30
Stress : 0.23
NMDS Axis 2
NMDS Axis 1

Fig. 5-6. Non-metric multi-dimensional scaling (NMDS) plots of non-target species in arthropod community assessed by sticky trap sampling in Autumn 2012 (A and B), Spring 2013 (C and D), and Autumn 2013 (E and F). Each symbol is a two-dimensional representation of non-target arthropod community. The distances between symbols were calculated by Bray-Curtis dissimilarity coefficient.

In all, 8,001 individuals belonging to 13 families of non-target arthropods were found by visual inspection (Table 4-3). Members of the Aphididae, Aleyrodidae, Chrysomelidae, Agromyzidae, and Noctuidae families accounted for more than 89% of that total. In 2013, *Harmonia axiridis* (Coccinellidae), *Phyllostreta striolata* (Chrysomelidae), *Liriomyza sp.* 1 (Agromyzidae), and *Eurydema gebleri* (Pentatomidae) were more abundant in Spring than in Autumn. *Nysius plebejus* (Lygaeidae) and *Trialeurodes vaporariorum* (Aleyrodidae) were found only in Autumn 2012. Meanwhile, the *Bt*-cabbage had more number of adult non-target herbivore, *Mamestra brassicae* than control cabbage in Spring 2013.

Table 5-3. Abundance (total number of individuals per cabbage line) of non-target arthropods in non-*Bt* (AD126) and *Bt* (C24 and C30) cabbage plots assessed by visual counts from Spring 2011 to Autumn 2013.

Taxon	Spring 2011			Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C24	C30	AD126	C30	AD126	C30
Coleoptera										
Cocinellidae										
Harmonia axyridis	1	0	0	0	0	0	13	10	3	0
Chrysomelidae										
Phyllotreta striolata	36	36	29	7	13	15	189	162	38	32
Diptera										
Agromyzidae										
Liriomyza sp.1	32	22	21	36	16	41	105	148	8	12
Hemiptera										
Berytidae										
Yemma exilis	0	0	0	0	0	0	9	12	0	0
Lygaeidae										
Nysius plebejus	0	0	0	1	4	3	0	0	111	66
Pentatomidae										
Eurydema gebleri	50	36	59	0	0	0	44	56	1	0
Miridae										
Apolygus spp	0	0	0	1	1	2	10	10	1	1
Homoptera										
Aleyrodidae										
Trialeurodes vaporariorum	0	0	0	272	357	174	0	0	0	0
Aphididae										
Aphididae spp	194	440	536	136	181	132	757	923	815	802
Hymenoptera										
Tenthredinidae										
Athalia rosae	0	0	0	0	0	0	0	0	16	7

Table 5-3. Continued.

Taxon	Spring 2011			Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C24	C30	AD126	C30	AD126	C30
Lepidoptera										
Noctuidae										
Mamestra brassicae	0	0	0	10	8	24	37	149	107	99
Araneae										
Theridiidae										
Theridiidae spp	2	8	6	13	6	8	8	10	2	4
Linyphiidae										
Linyphiidae spp	22	16	30	37	19	25	44	38	14	10

Throughout the entire three-year experimental period, more arthropod families were found through sticky-trap catches than by visual counts. Overall, 97,386 individuals belonging to 60 arthropod families were captured (Table 4-4). In this survey, large numbers of members within Aphididae (42.8% of the total), Thripidae (27.1%), Dolichopodidae (5.9%), and Chironomidae (5.4%) were collected. More arthropods were caught on traps in Spring than in Autumn.

Table 5-4. Abundance (total number of individuals during each study period) of non-target arthropods in non-*Bt* (AD126) and *Bt* (C24 and C30) cabbage plots assessed by sticky-trap sampling from Spring 2011 to Autumn 2013.

Taxon	Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C30	AD126	C30
Coleoptera							
Buprestidae							
Agrilus spp	0	0	0	86	53	0	0
Cantharidae							
Athemus vitellinus	0	0	0	0	2	0	0
Cantharis soeulensis	0	0	0	27	29	0	0
Chrysomelidae							
Lema decempunctata	0	0	0	1	1	0	0
Medythia nigrobilineata	0	1	1	1	0	0	0
Monolepta quadriguttata	1	1	0	0	0	0	0
Ophraella communa	0	0	0	5	4	0	1
Phyllotreta striolata	13	17	14	1216	994	31	35
Chrysomelidae spp	3	4	6	31	21	4	7
Coccinellidae							
Coccinella septempuntata	0	0	0	1	0	0	1
Harmonia axiridis	0	2	2	37	31	11	8
Hippodamia tredecimpunctata	0	0	0	3	4	0	1
Propylea japinica	0	0	0	18	23	2	2
Curculionidae							
Ceutorhynchus sp.1	0	0	0	461	378	0	0
Curculionidae spp	5	3	2	29	30	3	1
Dytiscidae							
Rhantus spp	3	6	2	0	0	0	0
Elateridae							
Aeoloderma agnata	0	0	0	17	11	1	1
Elateridae spp	0	0	0	0	0	0	0
Harpalidae							
Tachyura laetifica	8	8	9	21	18	7	12
Harpalidae spp	0	0	0	20	35	3	5
Mordellidae							
Mordellidae spp	0	0	0	24	22	0	0

Table 5-4. Continued.

Taxon	Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C30	AD126	C30
Staphylinidae							
Paederus fuscipes	1	1	0	5	1	2	1
Staphylinidae spp	84	19	44	113	129	214	119
Diptera							
Agromyzidae							
Liriomyza sp.1	30	22	9	50	27	48	32
Calliporidae							
Calliporidae spp	86	85	74	48	38	61	71
Chironomidae							
Chironomidae spp	642	744	500	911	1028	744	706
Cecidomyiidae							
Cecidomyiidae spp	100	126	138	352	355	49	39
Culicidae							
Culicidae spp	7	7	12	11	11	0	3
Dolichopodidae							
Dolichopodidae spp	464	374	440	1789	1917	363	448
Drosophilidae							
Drosophila sp.1	116	100	76	35	28	51	55
Lauxaniidae							
Lauxaniidae spp	4	2	4	17	16	19	23
Platystomatidae							
Rivellia apicalis	0	0	0	9	6	1	2
Rivellia nigroapicalis	3	2	6	7	9	10	8
Sarcophagidae							
Sarcophagidae spp	15	36	20	10	13	32	33
Sciomyzidae							
Sepedon aenescens	6	4	8	2	2	7	21
Sepsidae							
Sepsidae spp	11	3	11	8	6	0	0
Syrphidae							
Eristalomyia tenax	12	6	14	0	0	35	51
Syrphidae spp	74	86	54	9	11	54	60
Tephritidae							
Tephritidae spp	11	7	11	6	4	0	0
Tipulidae							
Tipulidae spp	6	9	9	9	13	17	19

Table 5-4. Continued.

Taxon	Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C30	AD126	C30
Hemiptera							
Anthocoridae							
Orius spp	32	21	19	121	161	30	13
Lygaeidae							
Geocoris varius	0	0	0	3	4	0	0
Nysius plebejus	35	50	54	89	79	418	399
Tropidothorax cruciger	0	0	0	4	2	0	0
Lygaeidae spp	2	8	27	2	1	2	0
Miridae							
Adelphocoris suturalis	29	10	15	98	66	16	15
Polymerus cognatus	0	0	0	34	22	4	5
Stenotus rubrovittatus	6	6	5	9	12	0	0
Apolygus spp	27	40	33	186	152	84	81
Miridae spp	17	27	19	124	93	29	30
Berytidae							
Yemma exilis	2	0	0	11	9	0	0
Alydidae							
Riptortus clavatus	3	0	2	0	0	0	0
Pentatomidae							
Dolycoris baccarum	1	0	0	2	1	1	0
Eurydema dominulus	0	0	0	38	40	0	0
Eurydema gebleri	0	0	1	219	282	8	7
Tingitidae							
Tingitidae spp	0	0	0	108	50	0	0
Homoptera							
Aphididae							
Aphididae spp	2960	2896	3196	11528	10802	5431	4886
Cicadellidae							
Bothrogonia japinica	0	0	1	2	2	0	0
Nephotettix cincticeps	0	0	0	6	7	0	0
Recilia dorsalis	8	9	7	7	11	15	20
Cicadellidae spp	83	72	55	91	89	21	10
Aleyrodidae							
Trialeurodes vaporariorum	59	62	59	0	0	0	0

Table 5-4. Continued.

Taxon	Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C30	AD126	C30
Delphacidae							
Delphacidae spp	31	33	48	57	69	88	112
Hymenoptera							
Apidae							
Apis mellifera	0	0	1	8	6	4	9
Braconidae							
Braconidae spp	188	162	152	265	208	456	474
Eulophidae							
Eulophidae spp	92	24	66	141	129	148	120
Formicidae							
Formicidae spp	2	0	0	1	0	0	0
Ichneumonidae							
Ichneumonidae spp	5	6	6	55	37	554	435
Tenthredinidae							
Athalia rosae	15	22	25	72	62	554	435
Lepidoptera							
Hesperiidae							
Parnara guttata	4	3	3	0	0	16	22
Noctuidae							
Mamestra brassicae	1	0	1	1	2	10	5
Noctuidae spp	1	4	1	0	0	1	0
Nymphalidae							
Polygonia c-aureum	0	10	0	0	0	0	0
Pyralidae							
Hymenia recurvalis	1	1	2	0	0	38	88
Neuroptera							
Chrysopidae							
Micromus angulatus	2	1	0	7	5	3	8
Chrysopidae spp	2	1	2	3	5	6	4
Odonata							
Libellulidae							
Sympetrum spp	1	1	0	0	0	9	8
Coenagrionidae							
Coenagrionidae spp	0	0	0	7	3	2	3

Table 5-4. Continued.

Taxon	Autumn 2012			Spring 2013		Autumn 2013	
	AD126	C24	C30	AD126	C30	AD126	C30
Orthoptera							
Acrididae							
Acrida cinerea	0	0	0	0	0	3	0
Aiolopus thalassinus	0	0	0	0	0	9	8
Oedaleus infernalis	0	2	2	0	0	4	2
Gryllidae							
Dianemobius nigrofasciatus	1	0	0	0	0	0	0
Tettigidae							
Tetrix japonica	3	1	4	0	0	0	0
Tridactylidae							
Xya japonica	0	1	2	10	9	5	5
Thysanoptera							
Thripidae							
Thripidae spp	464	666	446	11636	10513	1539	1163
Araneae							
Clubionidae							
Clubionidae spp	2	5	1	3	2	7	3
Linipidae							
Linipidae spp	11	8	9	12	7	0	1
Salticidae							
Evarcha albaria	0	0	0	1	2	1	1
Rhene atrata	0	0	0	3	3	1	1
Salticidae spp	11	3	2	1	4	2	1
Theridiidae							
Theridiidae spp	4	2	0	0	0	0	0
Thomisidae							
Thomisidae spp	0	1	0	2	0	1	2
Tetragnathidae							
Tetragnathidae spp	2	2	0	0	0	0	0

5.4. Discussion

As defined by Andaloro et al. (1983), our seedlings were transplanted at Growth Stage 2 (5-6 true leaves) and monitoring of target and non-target arthropods was conducted from Growth Stage 3 (6-8 true leaves) through Growth Stage 9, when harvesting occurred. This span covered most of the normal season of development for field-grown cabbage.

In this study, the abundance of *Plutella xylostella* was negatively influenced by *Bt* cabbage genotype, but the efficacy of the two transgenic lines differed. Whereas populations were consistently smaller on Line C30 than on AD126 in Spring 2011, Autumn 2012, and Spring and Autumn 2013, the effect associated with Line C24 was significant only in Autumn 2012. These results were consistent with the bioassay study conducted in the laboratory (Kim, 2014), which found the significantly greater mortality of *P. xylostella* larva fed on Line C30 than Line 24. We speculate that the Cry1Ac1 protein in C24 and C30 may have been differently expressed in the field. Kamble et al. (2013) have reported that expression levels of this protein can vary greatly among lines of transgenic Indian mustard (*Brassica juncea*), which then influences its toxicity to *P. xylostella.*

Other *Bt Brassica* crops expressing *cry1Ac* have been shown to control *P. xylostella* effectively in both the laboratory and the field. For example, *Bt* collard is linked with higher mortality of larvae under laboratory conditions (Cao et al.,

2005). Furthermore, larval survival and the average weight of individuals are significantly lower on *Bt* broccoli that expresses Cry1Ac protein (Tang et al., 1999). Finally, *Bt* canola expressing this protein effectively reduces the number of larvae and decreases the extent of damage to transgenic plants in both greenhouse and field (Ramachandran et al., 1998).

Cho et al. (2001) have shown that individuals of *Pieris rapae* are negatively affected on *Bt* Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) expressing Cry1C protein. Furthermore, Chen et al. (2008) have reported that *Bt* broccoli expressing this protein effectively manage those larvae, although later instars of that species are more tolerant of transgenic plants. Compared with the impact seen here by *Bt* cabbage on *Plutella xylostella*, those transgenics had less apparent effect on the abundance of *Pieris rapae*. In fact, the influence of genotype on that target was not statistically significant in Spring 2011 when assessed visually. However, counts were significantly reduced on Line C30 in Autumn 2012 and Spring and Autumn 2013. By contrast, when abundance was evaluated with sticky traps, no significant effects were found.

Sampling via traps in Autumn 2012 and Autumn 2013 did not reveal any variations in the numbers of *Plutella xylostella* and *Pieris rapae*, respectively, on transgenic versus non-transgenic lines, whereas visual counts indicated significant differences in abundance. This may have been due to the relatively small size of

experimental plots for monitoring of both species. Therefore, this trap method may be less efficient when monitoring cabbages in small field plots. Nevertheless, those traps proved effective for capturing very small, non-target insects, e.g., parasitic wasps, thrips, and species within *Orius*, which are not as easily identified during a visual survey. Thomson et al. (2004) have also shown that yellow sticky traps are suitable for monitoring small insects, such as Hymenopteran, Thysanopteran, and Hemipteran species.

We determined that the composition of non-target arthropod species in the field did not differ between *Bt* and non-*Bt* plants. Our data suggest that tested transgenic cabbage negatively affects only its target species. These findings are consistent with results reported from field studies of *Bt* poplars expressing Cry3A proteins (Zhang et al., 2011), *Bt* maize expressing VIP3A and Cry1Ab (Dively, 2005) or Cry1F proteins (Higgins et al., 2009), as well as *Bt* rice expressing Cry1Ab and Cry1Ac proteins (Li et al., 2007). However, Dively et al. (2004) showed a negative effect of a *Bt* (Cry1Ab) corn on a non-target butterfly, *Danaus plexippus*, in the field.

Cry1A toxin was originally designed for lepidopteran pest (Aronson and Shai. 2001), however, abundance of non-target lepidopteran herbivore, *Hymenia recurvalis* (Pyralidae), *Parnara guttata* (Hesperiidae), *Polygonia c-aureum* (Nymphalidae) and *Mamestra brassicae* (Noctuidae) were not affected by *Bt*

cabbage lines in the present study. *Hymenia recurvalis*, *P. guttata* and *P. c-aureum* do not feed on cabbage plant. Therefore, they will not be affected by *Bt* cabbage lines. Even *M. brassicae* population was more abundant than a control cabbage line in Spring 2013, which suggests that *M. brassicae* is tolerant to Cry1Ac1 toxin produced in our cabbages. Lightwood et al. (2000) reported that the *M. brassicae* is more tolerant to Cry1Ac toxin than *Pieris brassicae* (Lepidoptera: Pieridae).

Although research has been lacking about the impact of *Bt Brassica* crops on non-target arthropods in the field, feeding experiments have shown that *Bt Brassica* crops expressing Cry1Ac protein do not adversely affect non-target herbivores. Those investigations have included *Myzus persicae* on *Bt* cabbage (Nam et al., 2014), and *Mamestra brassicae*, or its parasitoid *Microplitis mediator* on *B. campestris* (Kim et al., 2008a). In addition, Howald et al. (2003) found that exposing a non-target herbivore, *Athalia rosae*, to *Bt* oilseed rape expressing Cry1Ac protein does not significantly affect mortality, growth, or fecundity of that insect. However, a potential hazard by *Bt*-transformed *B. campestris* that expresses Cry1Ac protein was reported by Kim et al. (2008b), who observed that high concentrations of pollen from those transgenics can significantly reduce the survival rate and growth of the non-target *Bombyx mori*. Nevertheless, that

research group also noted that the risk of exposure to such high pollen concentrations would be very unlikely under realistic field conditions.

5.5. Conclusions

In summary, we found that *Bt* cabbage expressing Cry1Ac1 protein can effectively control two Lepidopteran target pests, *Plutella xylostella* and *Pieris rapae*, in the field. Moreover, the composition of the non-target arthropod community does not differ between *Bt* and non-*Bt* cabbage plots. This implies that the presence of such transgenic plants has no significant impact on that community.

Acknowledgement

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5.6. References

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Chapter VI.

General Discussion

6.1. Overall Conclusions

This study aimed to reveal ecological risks of transgenic *Bt* cabbage on non-target arthropod species. It would provide assessment continuum within a tiered scheme of ecological risk assessment with the plans, results and discussion.

Our field monitoring experiments provide initial data on how exposure to Cry1Ac1 protein is reflected by measured concentrations in the bodies of arthropod herbivores, predators, and parasitoids in fields where transgenic cabbage is grown. Based on our results, Erigonidae predator species appears to be a competent surrogate species that can represent realistic scenarios of field exposure with *Bt* cabbage on non-target organisms. Moreover, we have identified arthropods that are directly or indirectly exposed to *Bt* toxin within the food web, and have shown how their degree of exposure varies over time during the growing season. The data in this study might be useful in establishing risk hypothesis to select appropriate tier-study. The transgenic cabbage can be exposure to non-target arthropod species.

Although numerous field studies have investigated how transgenic cotton and rice producing Cry1Ac proteins affect non-target arthropod communities (Liu et al. 2003; Naranjo 2005; Torres and Ruberson 2005; Han et al. 2014; Lee et al. 2014), less attention has been focused on measuring *Bt* protein concentrations and

examining tropically the consequences of *Bt* protein consumption (Wei et al. 2008; Torres and Ruberson 2007). As an extended laboratory study, our tritrophic assay showed that young wolf spiders grew normally from the third instar to adulthood when reared on fruit flies that had been fed either *Bt* cabbage or non-*Bt* cabbage. Although we detected the presence of Cry1Ac1 protein in the spider bodies, it did not affect their development and survival. Thus, cultivation of transgenic *Bt* cabbage does not influence the survival and growth of wolf spiders.

In the open field condition, we have identified that *Bt* cabbage expressing Cry1Ac1 protein can effectively control two Lepidopteran target pests, *Plutella xylostella* and *Pieris rapae*. Moreover, the composition of the non-target arthropod community does not differ between *Bt* and non-*Bt* cabbage plots. This implies that the presence of such transgenic plants has no significant impact on that community.

6.2. Further studies

I believe that the tiered risk study presented above would contribute to establish an environmental risk assessment that is essential to evaluate the possibility and risks of harm to non-target arthropods. However, for more detailed and validated test design, further studies are needed to develop such environmental risk studies.

1) Establishes risk hypothesis — Although monitoring of the levels of *Bt* proteins in arthropod species are well conducted in the field, we cannot collect the arthropod samples enough due to small plot size. In the case of small arthropods, such as Orius species, parasitic wasps and *Phyllotreta striolata* should collect relatively large amount of individuals to quantify the concentrations of *Bt* protein. Moreover, these conditions can be cause increasing between-plot interference. Therefore, a larger plot size is needed for such experimental field test. In addition, the data from more detailed cage study in the field will allow us to observe the food web interactions and the pattern of herbivores and natural enemy populations on transgenic crops and non-transgenic crops.

2) Earlier-tier study — an extended laboratory study provides complimentary information in specific endpoint under well controlled condition and ecological toxicity in tritrophic interaction. Our study showed the ecological effect of *Bt* cabbage on generalist predator, *P. astrigera* via tritrophic interaction. Although this study can also be simulated the degree of field exposure for the *Bt*

plant and food web realistically, actually, the primary consumer, *D. melanogaster* appears in the season-limited situation when the cabbage plants are decaying. Therefore, it is recommended to use *Bt*-resistant colony of target or non-target lepidopteran species that inhabit the *Bt* crop field as prey-test species. In this way, it is possible to provide fresh plant tissue to primary consumers, and there is no need to use artificially-processed (e.g. media, powder, etc.) and heated media mixed plant tissue. In addition, additional study that using another predator species, Erigonidae or Linyphiidae species strongly recommended. Those sedentary spiders make horizontally oriented webs and live at the base of several crop plants. Because of this, the crop plant and such spiders maintain a close relationship.

3) Higher-tier study — If any effects are detected in earlier-tier test at worst case exposure conditions, the risk can be leads to additional laboratory or higher-tier (open field) trials that reflects realistic *Bt* crop exposure scenarios (Romeis et al. 2008; 2011). As seen in our open field study, the composition of the non-target arthropod community does not differ between *Bt* and non-*Bt* cabbage plots. This result would imply that the presence of such transgenic plants has no significant impact on that community; however, actually, it can be difficult to determine the environmental risks with confidence. To complement this, the large plot size is necessary to encompass local populations of most arthropod

species in the community.

6.3. References

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적요

분자생물학적 기술의 급격한 발달을 통해 개발된 형질전환 작물은 해충, 제초제 등에 대한 저항성을 지닐 수 있게 되었고, 그로 인해 생산량의 증대, 생산단가의 절감 등의 획기적인 결과를 얻어낼 수 있기에 1996 년부터 상업적인 재배가 이루어지기 시작하였고, 매년 두 자릿수 이상의 재배면적 증가율을 기록하고 있다. 하지만 형질전환 작물이 생태계에 미칠 수 있는 잠재적 부정적인 영향에 대한 우려가 계속됨에 따라, 형질전환 작물의 상업화 이전에 과학적인 방법의 환경위해성 평가를 수행할 것을 바이오안전성 의정서와 국내 이행법인 형질전환 생물체의 국가간 이동 등에 관한 법률에서 명문화 하고 있다. 특히 형질전환 작물과 상호작용하는 생물체 중에서 일차소비자인 초식자와 이들을 먹이로 하는 상위단계의 포식자 및 기생자, 그리고 식물의 부식물질을 먹이로 하는 분해자가 모두 포함되는 절지동물 분류군은 작물에 의해 큰 영향을 받을 수 있으므로, 형질전환 작물이 절지동물에 미치는 영향을 평가하는 것은 위해성평가 과정 중 핵심적인 내용이다. 따라서 본 연구는 나비목 해충의 방제를 목적으로 한 형질전환 양배추의 절지동물을 이용한 환경위해성평가를 위한 접근을 다루고 있으며, 크게 세가지 연구단계에서 고찰하였다. (1) 실제 야외포장 환경 하에서의 절지동물 초식자와 포식자가 *Bt* 단백질에 노출되는 정도에 대한 모니터링. (2) 형질전환 양배추가 비표적 포식자인 별늑대거미의 생존과 성장에 미치는 영향. (3) 형질전환 양배추가 실제 야외포장 환경 하에서 표적곤충의 밀도와 비표적 절지동물의 군집구조에 미치는 영향. 형질전환 농작물은 식량문제, 과학기술의 발달, 그리고 사회적 인식에

따라 서로 다른 입장이 충돌할 수 있다. 본 연구가 이러한 근본적인 문제를 이해하는데 도움이 될 수 있을 것이다.

주요어: 형질전환 작물, 환경위해성평가, 절지동물, 생활사적 특성, 군집구조